Alanine-scanning Mutations in Domain 4 of Anthrax Toxin Protective Antigen Reveal Residues Important for Binding to the Cellular Receptor and to a Neutralizing Monoclonal Antibody*

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Bacillus anthracis secretes two toxins: edema toxin and lethal toxin. Each is composed of a common binding component, protective antigen (PA),1 together with an enzymatic component, edema factor (EF), in the case of edema toxin and lethal factor (LF) in the case of lethal toxin (1–3). The current model for toxin entry into the cell illustrates the centrality of PA for toxin action. PA binds to cellular receptors, recently identified as splice variants of either tumor endothelial marker 8 (TEM8) (4–6) or the closely related capillary morphogenesis protein 2 (CMG2) (7). Furin cleaves PA, releasing a 20-kDa fragment and a 63-kDa portion (PA63) capable of forming a heptamer, which has a newly exposed surface that binds EF and LF (8–12). Heptamer complexes enter the endocytic pathway by receptor-mediated endocytosis (13), and upon acidification and analytical ultracentrifugation. Residue Asp683 was the most critical for cell binding and toxicity, causing an ~1000-fold reduction in toxicity, but was not a large factor for interactions with 14B7. Substitutions in residues Tyr681, Asn682, and Pro686 also reduced toxicity significantly, by 10–100-fold. Of these, only Asn682 and Pro686 were also critical for interactions with 14B7. However, residues Lys684, Leu685, Leu687, and Tyr688 were critical for 14B7 binding without greatly affecting toxicity. The K684A and L685A variants exhibited wild type levels of toxicity in cell culture assays; the L687A and Y688A variants were reduced only 1.5- and 5-fold, respectively.

A panel of variants with alanine substitutions in the small loop of anthrax toxin protective antigen domain 4 was created to determine individual amino acid residues critical for interactions with the cellular receptor and with a neutralizing monoclonal antibody, 14B7. Substituted protective antigen proteins were analyzed by cellular cytotoxicity assays, and their interactions with antibody were measured by plasmon surface resonance and analytical ultracentrifugation. Residue Asp683 was the most critical for cell binding and toxicity, causing an ~1000-fold reduction in toxicity, but was not a large factor for interactions with 14B7. Substitutions in residues Tyr681, Asn682, and Pro686 also reduced toxicity significantly, by 10–100-fold. Of these, only Asn682 and Pro686 were also critical for interactions with 14B7. However, residues Lys684, Leu685, Leu687, and Tyr688 were critical for 14B7 binding without greatly affecting toxicity. The K684A and L685A variants exhibited wild type levels of toxicity in cell culture assays; the L687A and Y688A variants were reduced only 1.5- and 5-fold, respectively.

The development of new reagents to prevent and treat anthrax can be aided by our understanding of the molecular interactions between the toxin components and cellular targets as well as by interactions between the toxin components and potential reagents. One of the molecular reactions it would be beneficial to understand in molecular detail would be that between anthrax toxin and its cellular receptor. As noted above, much progress has been made in the study of PA and recently also with its cellular receptors, TEM8 and capillary morphogenesis protein 2, but studies of the interaction between the toxin with receptor have to date been limited to deletions or multiple substitutions in the proteins (6, 25). One goal of this study was to determine which individual amino acids in the previously identified region of PA were important for interaction with its receptor.

For development of improved vaccines and antibody-based...
Critical Residues in PA Domain 4

therapeutics, it would be helpful to identify the residues in PA necessary for recognition and neutralization of PA. 14B7 is one of a group of monoclonal antibodies that neutralize anthrax toxin by inhibiting the binding of PA to cells (26). Each antibody in this class recognizes an antigenic region between amino acids 671 and 721 of PA (17, 27). 14B7 and affinity-enhanced derivatives of it have been proposed as potential therapeutic reagents against anthrax (28). The overall goal of this work was to identify amino acids in the small loop region of PA domain 4 necessary for interaction with the neutralizing monoclonal antibody 14B7. Additionally, we sought to elucidate any overlap and differences between the amino acid residues necessary for PA binding to receptor and to 14B7.

EXPERIMENTAL PROCEDURES

Materials—The enzymes and reagents for DNA manipulations were from New England Biolabs (Beverly, MA), Panvera (Madison, WI), and Stratagene (La Jolla, CA). Mutagenic primers were purchased from the Center for Biologies Evaluation and Research (CBER) at the FDA (Bethesda, MD). Phenyl-Sepharose Fast Flow and Sepharcl S-100 were purchased from Amersham Biosciences. Wild type PA, LF, and FP59 were purified in our laboratory as previously described (29, 30). Rabbit anti-PA antibody (number 5308) was made in our laboratory, and reagents for the IgG-sepharose (26) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Monoclonal antibody 14B7 was purified from mouse ascites fluid.

Cell Lines and Culture Media—All culture media and cell culture solutions were purchased from Invitrogen. RAW264.7, a mouse macrophage cell line, was grown in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, 2 mM HEPES, 2 mM Glutamax I, and 50 mg/ml gentamycin. CHO cell clone 6 (CHO-CL6) and a furin-deficient cell line, previously described (34). Results were plotted and analyzed with the MTT to an insoluble blue pigment, which was measured as thiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT). Viable cells converted the MTT to an insoluble blue pigment, which was measured as previously described (34). Results were plotted and analyzed with Prism software (GraphPad Software Inc., San Diego) as the percentage viability of control wells containing LF without PA. EC_{50} values were determined by nonlinear regression sigmoidal dose-response analysis with variable slopes. Each assay was performed three times, and the graphs from a representative assay are shown in Fig. 1. Variant to wild type ratios of EC_{50} values were calculated.

CHO-CL6 cytotoxicity assays were performed similarly, with two exceptions: 1) the cells were plated at a density of ∼15,000 cells/well, because after the addition of the toxins (50 ng/ml FP59 with a dilution series of PA or PA variants from 0 to 2000 ng/ml), cells were incubated 48 h prior to MITT addition; and 2) FP59, a recombinant toxin consisting of the N-terminal PA-binding portion of LF fused to the ADP-ribosylation domain of Pseudomonas exotoxin A, was used in place of LF, because CHO cells are not lysed by lethal toxin. EC_{50} values were determined for three independent assays as above and are reported with S.E.

14B7 neutralization assays were also performed in 96-well plates in a similar manner as described above with RAW264.7 cells. In the 14B7 neutralization assays, PA or toxic PA variant and LF (200 ng/ml each) were preincubated for 30 min at 37 °C with 14B7 in a serial dilution from 0 to 10 μg/ml. Neutralization by rabbit polyclonal anti-PA antibody 5308 was tested by adding serum at a 1:800 dilution for preincubation with PA or toxic PA variant and LF (200 ng/ml each). For both types of assays (14B7 or polyclonal 5308), after the preincubation, the toxin/antibody solutions were added to cells from which medium had been aspirated and were incubated at 37 °C for 4 h. The MITT and analysis were identical to those in the cytotoxicity assays.

Assay of PA Binding to Cells—CHO FD11 cells were grown in 24-well plates to confluence. Cells were chilled to 4 °C in 0.5 ml of binding medium (minimal essential medium with Hanks’ salts, 1% fetal bovine serum, 10 mM HEPES). PA proteins were added to a concentration of 0.5 μg/ml, and incubation continued for 2 h. Cell monolayers were then washed five times with Hanks’ balanced salt solution (Biofluids, Rockville, MD) containing 1% fetal bovine serum. The cells were lysed in 100 μl of modified radioimmunoprecipitation lysis buffer (50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 2 mM MgCl_2, 10 units/ml benzamidine DNase (Novagen), and 1 tablet of Complete protease inhibitor (Roche Applied Science) per 20 ml. The cell lysates were added to SDS-PAGE gels (4–20% Tris-glycine gradient gels (Novex, San Diego, CA). Prior to loading, the lysates were boiled for 5 min in 1× SDS sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 100 mM dithiothreitol, 0.01% bromphenol blue, 6% glycerol). The proteins were then transferred to nitrocellulose membranes, followed by Western blotting. PA was visualized by chemiluminescence using the West Pico Kit (Pierce).

Surface Plasmon Resonance (SPR) Analysis—Bioreactor experiments were conducted with a Biacore X instrument. The antibody was covalently attached to a carboxyethyl dextran surface of an F1 chip using standard amine coupling (35), with the antibody dissolved at 0.08 mg/ml in 10 mM sodium acetate, pH 5.5. The reaction was stopped when 500–400 resonance units were cross-linked. Binding experiments were performed at 25 °C, in 10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.05% Tween 20, at a flow rate of 5 μl/min, and the surface was regenerated with 10 mM glycine, pH 2.0. For wild type PA and each variant, a series of association and dissociation sensorgrams were collected at different protein concentrations, starting at the stock concentration between 1 and 10 mg/ml and proceeding in serial dilutions by a factor 2 or 3 until no significant binding was detected. Each association assay was performed three times, and the data were fit to a single binding site model using a Langmuir isotherm (37) by nonlinear regression sigmoidal dose-response analysis with variable slopes.

Mathematical data analysis was based on a model with a heterogeneous population of surface sites with continuous binding constant distributions (36). In brief, the measured equilibrium surface binding signal s(c) was described as a superposition of binding signals s_k(c/κ_k) from different populations of sites with different affinities,

\[ s(c) = \int_{\kappa_{min}}^{\kappa_{max}} s_k(c/\kappa_k) P(\kappa_k)d\kappa_k \]  

(Eq. 1)

each following a Langmuir isotherm (37).

\[ s_k(c/\kappa_k) = 1/(1 + (c/\kappa_k)) \]  

(Eq. 2)

The differential affinity distribution function P(\kappa_k) was integrated over the peak of the high affinity sites to give a weight average estimate of the binding constant (36).

Critical Residues in PA Domain 4

30937
Analytical Centrifugation—Sedimentation equilibrium experiments were conducted in a Beckman Optima XL-I/A analytical ultracentrifuge (Beckman Coulter, Fullerton, CA). Antibody, wild type PA or variants, and mixtures were diluted in 150 μl of phosphate-buffered saline at loading concentrations of 0.1–0.3 mg/ml, and equilibrium absorbance profiles were acquired at rotor speeds of 9,000, 13,000, and 17,000 rpm; at a rotor temperature of 4 °C; and at wavelengths of 230, 250, and 280 nm. Protein extinction coefficients at 280 nm were estimated using the software SEDNTERP, kindly provided by Dr. J. Philo. Buoyant molar mass values for the proteins were obtained experimentally but were consistent with estimated values derived from amino acid sequence. Binding constants were calculated by global nonlinear regression with the software SEDPHAT, based on equations for the radial concentration distribution of ideally sedimenting species in mechanical and chemical equilibrium as described elsewhere (38, 39). The model for the analysis of the radial total protein distribution included four exponential terms, accounting for free antibody, free PA, and 1:1 and 1:2 complexes, linked at each radius by mass action law and the statistical factors for independent binding to two identical sites.

Sedimentation velocity experiments were performed at a rotor speed of 45,000 rpm and a temperature of 20 °C. Antibody, wild type PA or variants, and mixtures were diluted to a concentration of 0.2 mg/ml. 220 μl of PA solutions were filled in the sample sector of Epon double-sector centripieces, and mixtures of PA with antibody were filled in the reference sector. The light intensity transmitted through both sectors at 280 nm was measured with the absorbance scanning system in time intervals of 400 s. Using the software SEDFIT, the intensity data were transformed into pseudoabsorbance data (40) and analyzed with the model of diffusion-corrected sedimentation coefficient distributions, c(s) (41-42). As a result, separate sedimentation coefficient distributions for the PA and the mixtures were obtained, which resolved free PA, antibody, and the complex. Interpretation of the sedimentation coefficient distributions directly allowed both the determination of the active concentration of PA variants and the semiquantitative assessment of the strength of the interaction between PA and the antibody from the sedimentation coefficient of the complex.

RESULTS

Several Residues in the Small Loop Region of PA Domain 4 Are Important for Toxicity—Previous studies in our laboratory with multiple substitutions in PA domain 4 indicated that the small loop region was critical for binding to the anthrax receptor (25). To determine the importance of individual residues, we made a set of alanine-scanning mutants covering the domain 4 small loop and including residue Asn^657, which is outside the small loop but located near in the crystal structure. In a few cases, we made doubly or triply substituted proteins to ascertain if effects would be additive or cooperative. Initial assessments of binding ability were made utilizing the mouse macrophage RAW264.7 cell line, which rapidly lysed upon the addition of PA and LF. If the PA variants failed to bind, the cells would be unaffected by the addition of LF and would metabolize the MTT dye similarly to untreated controls, forming a blue pigment. Variants that successfully bound to the receptor would transport LF, causing cell lysis and the inability to convert MTT to a blue pigment. The assay was performed three times, with results from a representative assay shown in Fig. 1A. The RAW264.7 assay exhibited slightly variable EC_{50} values from day to day, but the ratios of the substituted proteins to wild type remained constant. The range of ratios for PA variant/wild type EC_{50} values calculated from the three independent assays is reported in Table I.

PA variants D683A, N657A/N682A, D683A/L685E/Y688C, and D683A/L685E/Y688K were all nontoxic at 1 μg/ml, the highest level tested in the assay (Fig. 1A). Several amino acid substitutions shifted the EC_{50} value from that of wild type toxin, 13 ng/ml. The N682A variant exhibited such low toxicity in this assay that it was not possible to determine an EC_{50} value (Fig. 1A and Table I). Substituting alanine for residues Pro^686 and Tyr^681 also had large effects on toxicity, making the variants 9- and 11-fold less toxic than wild type PA, respectively (Table I). Variants N657A, L687A, and I689A were about 5-fold less toxic than wild type. A smaller effect on toxicity was seen for the K680A variant, which was 2-fold less toxic than PA. The remainder of the substitutions did not appear to affect toxicity and had variant/wild type EC_{50} ratios of 1.

To due to the variability and limited sensitivity of the cytotoxicity assays with RAW264.7 cells, the panel of variants was also assayed in a CHO cell line, CHO-CL6. Although CHO cells do not undergo lysis upon the addition of PA and LF, they do express 10,000 PA receptors/cell (43). To circumvent the insensitivity of CHO cells to LF, in its place we used FP59, a fusion toxin between the N-terminal portion of LF (residues 1–254) and the ADP-ribosylation domain of Pseudomonas exotoxin A. Such fusions are extremely toxic to CHO cells when applied with PA (44). EC_{50} values for wild type PA were lower for CHO cells than for the RAW264.7 cell line (Fig. 1, compare A and B), making the CHO-CL6 cytotoxicity assay more sensitive than the RAW cell assay. EC_{50} values for the wild type protein and the panel of variants are reported in Table I.

The pattern of toxicity levels for the variant panel was similar in CHO-CL6 cell assays to that in RAW264.7 cell assays (Table I), but the increased sensitivity of the CHO-CL6 assay provided further information. It was possible, for instance, to determine an EC_{50} value for the N682A protein, which was not possible in the assay with RAW264.7 cells. This substitution decreased toxicity almost 150-fold. The D683A variant also exhibited weak toxicity in tests with CHO-CL6 cells, and in one of the three assays there were sufficient data points to determine an EC_{50} value of 828. This protein seems to fall at the limit of EC_{50} determination, because at protein levels higher than 2 μg/ml (the highest level tested in this assay), nonspecific uptake of toxins becomes a concern, and toxicity in this case would not reflect receptor-PA interaction.

The N657A/N682A doubly substituted protein did not exhibit any toxicity at 2 μg/ml in either cell culture system, whereas single alanine substitutions at these residues decreased toxicity by 7- and 140-fold, indicating that effects of the substitutions were synergistic. The two variants triply substituted at residues Asp^683, Leu^685, and Tyr^688 also remained nontoxic for CHO-CL6 cells. The triple variants were interesting because the singly substituted D683A protein did exhibit some toxicity at high concentrations in the CHO-CL6 cytotoxicity assay, and neither the L685A nor Y688A single substitutions greatly affected toxicity. It may be that the non-alanine amino acid substitutions for Leu^685 and Tyr^688 in the triple variants had greater effects on binding ability than the alanine substitutions. It is also possible that 1.5-fold loss of toxicity with the single amino acid substitution at position Tyr^688 was additive in the D683A background.

Variants Y681A and P686A were 32- and 17-fold less toxic than PA in the assay with CHO-CL6 cells, distinguishing between the similar values (9 and 11, respectively) obtained in the RAW264.7 assay (Table I). Single amino acid substitutions N657A, L687A, and I689A again were grouped closely together, exhibiting 6-, 5-, and 4-fold losses of toxicity, respectively. Results for the K680A variant were very similar to those in the assay with RAW264.7 cells, with a 2-fold reduction in toxicity levels.

Decreases in Toxicity Are Due to Decreases in Cell Binding—Based on previous studies in our laboratory, it was likely that the decreased cytotoxicity in the cell culture assays was due to the decreased ability of the variants to bind to the cellular receptor. To rule out the possibility that the less toxic variants bound cells similarly to the wild type protein and exhibited a loss in toxicity for other reasons, we assessed the ability of the proteins to bind to CHO FD11 cells. CHO FD11 cells are deficient in furin, so this cell line provided a background in which
receptor binding by the variants was investigated without allowing further processing. The variants were allowed to bind to CHO FD11 cells for 2 h at 4 °C. After washing to remove any unbound protein, cell lysates were prepared, subjected to SDS-PAGE, and transferred to nitrocellulose for Western blot analysis with polyclonal anti-PA serum. Fig. 2 shows that wild type PA bound strongly to cells. The results of the binding assay correlated very well with the toxicity assays. None of the less toxic variants bound to cells in amounts similar to the wild type toxin. The K680A and Y688A substitutions caused small decreases in toxicity and likewise bound more similarly to wild type PA than other substitutions that had more severe toxicity defects. N657A, L687A, and I689A bound more poorly in correlation with their greater defects in toxicity; binding by the nontoxic variants was barely detectable. As expected, the toxic variants retained the ability to bind to cells in levels similar to that of wild type protein.

Several Small Loop Residues Are Essential for 14B7 Neutralization—Monoclonal antibodies that block PA binding to cells have been isolated and shown to bind to domain 4 of PA (17, 26). From our cell binding and toxicity assays, it was clear that several amino acids in the small loop region of domain 4 are required for PA binding to cells, and it was conceivable that the same region of PA could also be recognized by the monoclonal antibodies blocking PA binding to cells. We tested this idea with one such neutralizing antibody, 14B7. For these assays, only toxic variants could be tested. In the assay, 200 ng/ml each of LF and PA variant were preincubated with varying amounts 14B7 from 0 to 10 μg/ml prior to the addition to RAW264.7 cells. The ability of 14B7 to protect the RAW cells from lysis was then assayed with MTT as in the previous cytotoxicity assays. Results from a representative assay are shown in Fig. 3.

In stark contrast to wild type PA, singly substituted PA variants K684A, L685A, L687A, and Y688A were not neutralized by 14B7, even when antibody was added in 25-fold molar excess (10 μg/ml) over the variants (Fig. 3A). Of the four substitutions rendering the variants resistant to 14B7, K684A and L685A had no measurable effect on toxicity. L687A reduced toxicity 5-fold, and Y688A consistently exhibited a slightly reduced level of toxicity. Four other single substitutions in PA had decreased neutralization by 14B7; variants K679A, I689A, S690A, and N691A required greater concentrations of 14B7 for neutralization than did wild type PA (Fig. 3B). Of those substitutions affecting 14B7 neutralization, only L687A significantly affected toxicity. The remaining variants were neutralized by levels of 14B7 similar to the levels that neutralized wild type protein (Fig. 3C). Variants Y681A and P686A were already less toxic than wild type at the levels tested in this assay (200 ng/ml), so their neutralization may not reflect the same level of binding needed for neutralization of the other variants and wild type protein. It should be noted that only variants
exhibiting significant toxicity could be tested for neutralization, so this assay was not useful for interpreting the contributions of residues Asn682 and Asp683 to interaction with 14B7. Resistance to antibody neutralization was specific to 14B7; all toxic variants were completely neutralized by polyclonal anti-PA serum (data not shown).

*Biophysical Measurements Reveal That Residue Asp683 Is Not Essential for 14B7 Binding*—Equilibrium constants of the antibody binding to the different variants were determined independently by SPR, sedimentation equilibrium, and sedimentation velocity. Sedimentation equilibrium experiments provide the most direct measure for the equilibrium constants and gave results for the wild type interaction that are consistent with the affinity previously reported (28) (Fig. 4, *top panel*). Unfortunately, the extended time necessary to attain sedimentation equilibrium at different rotor speeds, combined with the rigorous requirements for sample purity did not permit sedimentation equilibrium experiments with all variants. Sedimentation velocity provided a measure for the relative affinities through comparison of the populations of protein migrating at the sedimentation rate of free PA, free antibody, and with the complex, all of which can be resolved in the diffusion-corrected sedimentation coefficient distributions. Because the reversible binding reaction is taking place on the time scale of the sedimentation experiment, an indirect relative measure of the binding constant could be derived from a single sedimentation experiment by comparing the sedimentation rate of the complex (Fig. 4, *lower panel*). However, this measure is a combined measure of the rate and affinity constants of the interactions. In addition, the hydrodynamic separation of the protein from peptides and aggregates allowed the determination of the active PA concentration. This information was used for the following SPR analysis.

The analysis of the kinetics of PA binding to immobilized antibody revealed that the measured surface binding kinetics in the SPR biosensor was transport-limited for the wild type PA and most of the high affinity variants. An analysis of the binding progress in the presence of transport limitation can be very difficult, because it requires assumption about, and modeling of, the detailed reaction/diffusion process at the sensor surface that is governing the measured surface binding. Nevertheless, the empirically observed rate constants of the surface binding signal can be taken as lower limits for the chemical rate constants. (The true chemical rate constants may be higher and masked by the transport being the rate-limiting step, but they cannot be slower than the observed apparent rate constants.) For the wild type, the minimal estimates of the on-rate and off-rate constants were as follows: $k_{\text{on}} > 0.003/s$, ...

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**Table I**

Properties of domain 4 small loop region variants

| Variant | RAW266.7 EC_{50} | CHO-CL6 EC_{50} | 14B7 neutralization | $K_D$ |
|---------|------------------|-----------------|---------------------|------|
| WT      | 1                | $1.02 \pm 0.16$ | ++                  | 6 nM |
| N657A   | 3-8              | $6.59 \pm 0.59$ | ++                  | 46 nM |
| K678A   | 1-2              | $1.02 \pm 0.08$ | +                   | 46 nM |
| K680A   | 2-3              | $1.82 \pm 0.15$ | ++                  | 45 nM |
| Y681A   | 9                | $31.9 \pm 2.6$  | ++                  | 58 nM |
| N682A   | NA \(^f\)       | $143 \pm 4.7$   | NA                  | 450 nM |
| D683A   | NT \(^e\) at 1 \(\mu\)g/ml | 828 \(^a\) | NA                  | 52 nM |
| K684A   | 1                | $0.871 \pm 0.09$ | –                   | 106 nM |
| L685A   | 1                | $0.832 \pm 0.05$ | –                   | >6 nM |
| P686A   | 11-12            | $17.4 \pm 1.2$  | ++                  | 155 nM |
| L687A   | 4-7              | $5.04 \pm 0.54$ | –                   | 405 nM |
| Y688A   | 1-2              | $1.52 \pm 0.21$ | –                   | 887 nM |
| I689A   | 4-8              | $3.98 \pm 0.21$ | +                   | 82 nM |
| S690A   | 1                | $0.989 \pm 0.21$| +                   | 44 nM |
| N691A   | 1                | $1.06 \pm 0.20$ | +                   | 31 nM |
| P692A   | 1                | $1.09 \pm 0.22$ | +                   | 34 nM |
| N693A   | 1                | $0.903 \pm 0.07$| ++                  | <10 nM |
| N657A/N682A | NT at 1 \(\mu\)g/ml | NT at 2 \(\mu\)g/ml | NA                  | 1.2 \(\mu\)M |
| D683A/L685E/Y688C | NT at 1 \(\mu\)g/ml | NT at 2 \(\mu\)g/ml | NA                  | 10 \(\mu\)M |
| D683A/L685E/Y688K | NT at 1 \(\mu\)g/ml | NT at 2 \(\mu\)g/ml | NA                  | 5 \(\mu\)M |

\(^a\) WT, wild type.

\(^b\) Values and S.E. reported for three assays, except when noted otherwise.

\(^c\) Determined by sedimentation equilibrium.

\(^d\) Determined by SPR.

\(^e\) NA, not applicable.

\(^f\) Although variant exhibited some toxicity at 1 \(\mu\)g/ml, EC_{50} value could not be determined.

\(^g\) NT, nontoxic.

\(^h\)Replicate experiments were performed; however, only one assay provided a valid EC_{50}.
Critical Residues in PA Domain 4

Fig. 3. Several substitutions reduce the ability of 14B7 to neutralize PA. RAW264.7 cells were incubated with 200 ng/ml PA and LF preincubated (30 min) with increasing amounts of 14B7 spanning from 0 to 10 μg/ml. Cells plus toxin and antibody were incubated for 4 h at 37 °C followed by staining for viability with MTT as in Fig. 1. Results were plotted as the percentage of viable cells compared with controls without PA added. Each panel contains wild type PA for comparison, A, PA substitutions that eliminated 14B7 neutralization; B, PA substitutions that reduced 14B7 neutralization; C, PA substitutions neutralized by 14B7.

and $k_{\text{on}} > 5 \times 10^9$/ms (Fig. 5, top). Because of these difficulties in the analysis of the binding kinetics, the quantitative comparison of the binding properties of the variants was essentially restricted to the determination of the equilibrium binding constant from the interpretation of the equilibrium isotherms (Fig. 5, middle). When using a standard CM5 dextran-coated sensor chip, artifactual binding sites with ~100-fold lower affinity were observed that dominated the surface binding. The generation of lower affinity sites has been commonly observed when using the random immobilization chemistry, and similar effects have been attributed also to steric hindrance and/or influences of the local microenvironment in the immobilization matrix (45). With the short dextran layer of the F1 chip, the low affinity sites amounted to only ~50% of all sites, and they could be discriminated from the higher affinity PA binding sites that reflected the unimpeded interaction, as judged by consistency of those binding constants with sedimentation equilibrium results (Fig. 5, bottom).

Thus, each of the biophysical techniques applied exhibited specific limitations for the characterization of some PA variants. However, for the variants where the methods overlap, the binding constants from the high affinity surface binding site, from sedimentation equilibrium, and from the semiquantitative ranking by sedimentation coefficient distributions gave consistent results (with affinity constants by ultracentrifugation and SPR generally within a factor of 2–3 or better). The combination of the results from the three techniques provided a complete description of the affinity of the variants (Table I, far right column). From these data, it was possible to distinguish a set of single amino acid substitutions that had a large effect on affinity to antibody, including L685A, which reduced the affinity to 14B7 at least 10,000-fold compared with wild type PA. Three individual substitutions reduced binding affinity ~70–100-fold: N682A, L687A, and Y688A. Substitutions

Fig. 4. Characterization of the affinity of the PA variants by analytical ultracentrifugation. Top, sedimentation equilibrium profiles of mixtures of 14B7 antibody with wild type PA (solid line), S690A (dashed line), P686A (dotted line), and L685A (dashed and dotted line) at 9,000 rpm. In this assay, increasing complex formation is indicated by increasing curvature and steepness of the profiles. For clarity, the data shown are part of complete sedimentation data acquired for each variant at multiple rotor speeds and detection wavelengths, subjected to global mathematical analysis for determination of the equilibrium constant. $K_D$ values obtained are ~6 nM for wild type, 44 nM for S690A, and >64 μM for L685A, respectively. The equilibrium constant for P686A could not be determined by this method. Lower panel, sedimentation coefficient distributions of mixtures of 14B7 with wild type PA (solid line), S690A (dashed line), P686A (dotted line), and L685A (dashed and dotted line). For comparison, the sedimentation coefficient distributions are shown of wild type PA alone (+), and 14B7 alone (×). The sedimentation coefficient of the wild type PA-14B7 complex is highest, due to the highest affinity and stability. For L685A, virtually no complex is formed, and the peaks in the sedimentation pattern are virtually like those measured for the separate proteins. PA P686A shows a broader distribution with some complex and some free 14B7. The inset shows pseudoabsorbance versus radius at different times for the original velocity sedimentation data of the PA P686A/14B7 mixture, corrected for systematic noise components.
K684A and P686A reduced affinity 18- and 26-fold, respectively. Where measurable by neutralization assays, this group of six single substitutions prevented 14B7 neutralization, with the exception of P686A, which exhibited greatly reduced toxicity compared with wild type protein (Fig. 3, compare wild type and P686A traces at low levels of 14B7) and so could most likely be neutralized more easily than more toxic variants tested by neutralization. Other single amino substitutions had less effect on affinity. N693A exhibited a \( K_D \) value similar to that of wild type. The remaining singly substituted variants had \( K_D \) values between 6- and 14-fold higher than wild type. This class of variants was at least partially neutralized by 14B7 for variants that were toxic enough to be tested in that assay. Interestingly, substitution D683A falls into this category with a \( K_D \) value 9-fold higher than wild type PA. Whereas the D683A substitution prevented toxicity (and hence neutralization testing), the biophysical measurements indicated that it did not greatly affect 14B7 binding affinity. When the binding constant of the double mutant N657A/N682A was compared with those of the single mutants, within the error limits of the experiments, no significant thermodynamic cooperativity of these residues for 14B7 binding was found.

**DISCUSSION**

The binding of PA to its cellular receptor is essential for anthrax toxin activity. One way to prevent anthrax toxicity is by blocking this step. In order to do so, it would be helpful to understand at the molecular level the residues of PA that interact with the cellular receptors and with antibodies that block PA cellular binding. For this study, we created a set of alanine-scanning mutants covering the small loop of PA domain 4 to elucidate amino acid residues important for binding to cellular receptors and to a neutralizing antibody, 14B7. These studies revealed that the PA domain 4 small loop residues most important for toxicity overlapped with, but were distinct from, the set of residues most important for 14B7 binding. This finding is illustrated in Fig. 6, where the substitution of residues shown in red (Tyr\(^{681}\) and Asp\(^{683}\)) or, to a lesser extent, pink (Asn\(^{657}\)), created large defects primarily in cell binding and toxicity; substitutions of residues shown in green (Lys\(^{684}\), Leu\(^{685}\), Leu\(^{687}\), and Tyr\(^{688}\)) greatly decreased or eliminated 14B7 binding with milder effects on cell binding and toxicity; and substitutions of residues shown in yellow (Asn\(^{652}\) and Pro\(^{686}\)) eliminated or greatly reduced binding to both cells and 14B7. It should be noted that the color coding in Fig. 6 is distinct from, the set of residues most important for 14B7 binding. This finding is illustrated in Fig. 6, where the substitution of residues shown in red (Tyr\(^{681}\) and Asp\(^{683}\)) or, to a lesser extent, pink (Asn\(^{657}\)), created large defects primarily in cell binding and toxicity; substitutions of residues shown in green (Lys\(^{684}\), Leu\(^{685}\), Leu\(^{687}\), and Tyr\(^{688}\)) greatly decreased or eliminated 14B7 binding with milder effects on cell binding and toxicity; and substitutions of residues shown in yellow (Asn\(^{652}\) and Pro\(^{686}\)) eliminated or greatly reduced binding to both cells and 14B7. It should be noted that the color coding in Fig. 6 reflects only the most extreme defects. For instance, the L687A variant did exhibit a reduction in binding and toxicity, but the reductions were not as great as those seen for the substitutions in residues Asp\(^{683}\), Tyr\(^{681}\), and Asn\(^{657}\). Additionally, the Y689A variant was neither fully toxic nor fully neutralized, but again
the defects were less pronounced than those for substitutions of residues highlighted in Fig. 6.

The results from the 14B7 binding and neutralization assays were very interesting, because it was apparent from the biophysical measurements that with the exception of the N693A substitution, all of the singly substituted variants had at least a 5-fold weaker binding to 14B7 (Table I). However, only larger changes correlated with the loss of biological activity as seen by the neutralization assay. It may be that in addition to the large contributions made by the residues highlighted in Fig. 6, many residues in the small loop make small contributions to PA-14B7 interaction and that disrupting one such small contribution does not prevent productive binding. The additive nature of multiple changes in reducing binding affinities can be seen in the doubly substituted N657A/N682A variant. The singly substituted N657A variant exhibited an 8-fold reduction in binding affinity, and the singly substituted N682A variant showed a 75-fold reduction. The double N657A/N682A substitution caused a 200-fold loss in binding affinity (Table I). Although additive, however, these residues did not exhibit a significant cooperativity, suggesting that their free energy contribution to antibody binding is largely independent. The two triply substituted variants also had very low binding affinities for 14B7, but in this case it is difficult to determine whether any additional effect was seen over that of the single Leu685 substitution, which alone completely disrupted binding.

It is apparent from our results that several small loop residues are critical for productive 14B7 binding and that other small loop residues also contribute to the binding affinity. It is possible that there are other residues involved in 14B7 binding that were not covered by our collection of PA variants. Previous studies localized the 14B7 epitope to a region between PA residues 671 and 721 (17), so our collection falls within this region but does not completely cover it. It is important to note that whereas some substitutions completely prevented neutralization by monoclonal antibody 14B7, all of the variants studied in this work were neutralized by polyclonal serum, indicating that the changes affected the ability of the 14B7 to block receptor binding but did not affect other PA epitopes contributing to protection by polyclonal serum.

The cell binding and toxicity assays implicated D683 as a critical residue in receptor binding. This finding is consistent with an interaction between the carboxyl group of Asp683 and the metal coordination site of the anthrax receptors, TEM8 and CMG2. Recent studies in our laboratory have shown that the extracellular domain and a transmembrane domain or membrane anchor are necessary for PA activity, whereas the cytoplasmic tail is not essential (6). The extracellular portions of the receptors contain a von Willebrand factor type A domain with a metal ion-dependent adhesion site (MIDAS) motif. Thus, although the physiological functions of the receptors remain unknown, some things can be surmised about TEM8 and CMG2 (269, 385, 833, –10277 to –10281).

Understanding the relevance of individual residues in PA to binding interactions with TEM8 or CMG2 and with neutralizing antibodies provides useful information for developing anthrax therapeutics. The residues identified in this work may provide targets for directed antitoxins. Additionally, the results of individual amino acid changes in PA disrupting neutralization by 14B7 imply that monoclonal antibody therapies should include a mixture of antibodies to different PA epitopes. Additionally, the PA variant panel used in this study could be useful in evaluating potential therapeutics directed against the binding domain of PA.

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Alanine-scanning Mutations in Domain 4 of Anthrax Toxin Protective Antigen Reveal Residues Important for Binding to the Cellular Receptor and to a Neutralizing Monoclonal Antibody

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