The protective antigen (PA) moiety of anthrax toxin binds to cellular receptors and mediates entry of the two enzymatic moieties of the toxin into the cytosol. Two PA receptors, anthrax toxin receptor (ATR)/tumor endothelial marker 8 (TEM8) and capillary morphogenesis protein 2 (CMG2), have been identified. We expressed and purified the von Willebrand A (VWA) domain of CMG2 and examined its interactions with monomeric and heptameric forms of PA. Monomeric PA bound a stoichiometric equivalent of CMG2, whereas the heptameric prepore form bound 7 eq. The K_d of the VWA domain-PA interaction is 170 pM when liganded by Mg^{2+}, reflecting a 1000-fold tighter interaction than most VWA domains with their endogenous ligands. The dissociation rate constant is extremely slow, indicating a 30-h lifetime for the CMG2-PA monomer complex. CMG2 metal ion-dependent adhesion site (MIDAS) was studied kinetically and thermodynamically. The association rate constant (10^5 M^{-1} s^{-1}) is virtually identical in the presence or absence of Mg^{2+} or Ca^{2+}, but the dissociation rate of metal ion liganded complex is up to 4 orders of magnitude slower than metal ion free complex. Residual affinity (K_d ~ 960 m\text{n}) in the absence of divalent metal ions allowed the free energy for the contribution of the metal ion to be calculated as 5 kcal mol^{-1}, demonstrating that the metal ion-dependent adhesion site is directly coordinated by CMG2 and PA in the binding interface. The high affinity of the VWA domain for PA supports its potency in neutralizing anthrax toxin, demonstrating its potential utility as a novel therapeutic for anthrax.

The pathology of the anthrax bacillus, Bacillus anthracis, is due in part to the production of anthrax toxin, an ensemble of three nontoxic monomeric proteins that combine at the surface of host cells to form toxic noncovalent complexes (see Fig. 1A). Two of these proteins are enzymes that modify cytosolic substrates. Lethal factor (LF, 90 kDa) is a Zn^{2+} protease that cleaves several mitogen-activated protein kinase kinases (1, 2), and edema factor (EF, 89 kDa) is a Ca^{2+}- and calmodulin-dependent adenylyl cyclase (3). The third protein, protective antigen (PA83, 83 kDa), binds to cellular receptors and transports LF and EF to the cytosol.

The initial step in the action of the toxin is the binding of PA to a cell surface receptor. Receptor-bound PA is cleaved into two fragments by a furin family protease (4). Dissociation of the smaller fragment allows the larger fragment, which remains receptor-bound, to self-associate into ring-shaped heptamers (PA_{63}), also referred to as prepore (5). Prepore may then bind up to three molecules of LF and/or EF with nanomolar affinity (6, 7). The resulting complexes are endocytosed to an acidic compartment (8, 9), where the heptamers are converted from the prepore state to an integral membrane, ion-conductive pore (10). The process of translocating LF and EF into the cytosol is linked to the formation of pore, but the nature of this linkage is poorly understood. Within the cytosol these enzymatically active moieties may then disrupt normal cellular physiology.

Two anthrax toxin receptors, CMG2 (11) and anthrax toxin receptor (ATR)/tumor endothelial marker 8 (12), are known. Each is a single peptide chain consisting of an extracellular domain, a membrane-spanning region, and a cytoplasmic tail. In their extracellular domains, there is an ~200-amino acid von Willebrand type A (VWA) domain that shows 60% amino acid identity between the two proteins (11). This domain adopts a dinitocleotide binding or Rossmann fold that is composed of a sandwich of six eight amphipathic a-helices that surround a hydrophobic 4-sheet (see Fig. 1B). The VWA domain fold is found in many cell adhesion proteins and generally promotes protein-protein interactions (13). Many VWA domains contain a highly conserved metal ion-dependent adhesion site (MIDAS) that is often involved in ligand interactions (14). The metal ion adopts an octahedral geometry and is coordinated by residues from three of its loops as well as two to three ordered water molecules. Usually, a glutamic or aspartic acid side chain from the ligand completes this metal ion coordination sphere; therefore, the metal ion acts as a bridge between the ligand and VWA domain. Consistent with a metal ion-mediated interaction, both CMG2 and ATR have been shown to bind PA more tightly in the presence of divalent cations (11, 12).

Here, we quantify the binding interaction of soluble CMG2 VWA domain with PA_{63} monomer and PA_{63} heptamer. Mono-
meric PA bound a stoichiometric equivalent of CMG2, whereas the heptameric prepore form bound 7 eq. The equilibrium dissociation constant ($K_d$) for CMG2-VWA domain interaction with monomeric PA$_{3}$3 is very tight (170 µM), and the dissociation rate constant is extremely slow ($\sim 10^{-5}$ s$^{-1}$). We show that the tight binding affinity relies on the presence and identity of the divalent MIDAS metal ion. Knowledge of the affinity and slow dissociation rate of CMG2-PA complexes supports the notion that CMG2 VWA domain may be used clinically as an inhibitor of anthrax toxin.

**Materials and Methods**

**Plasmid Construction**—A DNA sequence encoding residues 35–225 of the CMG2 VWA domain (referred to as CMG2$^{35-225}$) was cloned into pGEX4T1 (Amersham Biosciences) using 5′ BamHI and 3′ NotI restriction sites. pGEX4T1 includes a thrombin-cleavable glutathione S-transferase tag on the amino terminus of the expressed protein. Two truncated versions of CMG2 were then generated using PCR and the same 5′ BamHI and 3′ NotI sites, (i) residues 38–218 (CMG2$^{38-218}$) and (ii) residues 40–217 (CMG2$^{40-217}$). The latter version eliminated the natural disulfide bond. To generate a version of CMG2 with a single, unique cysteine moiety point mutation on the amino terminus, two successive rounds of site-directed mutagenesis were performed on CMG2$^{38-218}$. The CMG2$^{38-218}$C175A variant was introduced to eliminate a buried cysteine, and the R40C mutation created a unique cysteine residue on the more accessible amino terminus, making CMG2$^{40-217}$.

**Preparation of Proteins**—Recombinant lethal factor amino-terminal domain (residues 1–263; LF$_{54}$) was purified as described previously (15). Recombinant PA was expressed in BL21(DE3) using pET22b-PA (Novagen), which directs expression to the periplasm. Gram-scale expression of PA was carried out in a 1-liter Bioflo 110 fermenter (New Brunswick Scientific). Using ECMP1 growth media (16), cells were harvested by the computer A/D card. During acquisition, the computer automatically every 15 s for 7 min. All experiments were performed using the Biacore 2000 system sensor chips. For CM5 chips, the system was maintained at a constant flow rate of 5 µl min$^{-1}$ of HBS buffer (10 mM HEPES, pH 7.4, 150 mM NaCl) at 25 °C. Monomeric PA$_{3}$3 was covalently linked to the carboxylated dextran matrix. Monomeric PA$_{3}$3 was diluted to 2 µM in sodium acetate buffer, pH 4.5. The protein was injected onto the activated surface at a flow rate of 5 µl min$^{-1}$ until the desired base-line level of 2000–4000 response units was obtained (−5 min) and then blocked with ethanolamine.

CMG2 VWA domain variants were diluted into HBS running buffer with either 1 mM CaCl$_2$ or 1 mM CuCl$_2$ plus 0.5 mM tris-(2-carboxyethyl)phosphine, pH 7.4, and serial injections were made at 10 µl min$^{-1}$. Concentrations of protein ranged from 47 nM to 4.8 µM. CM5 base lines were regenerated with a 30-µl pulse of 0.5 mM NaClO$_3$, pH 10.5, resulting in <1% loss of base line per injection.

**Isothermal Titration Calorimetry—**Experiments were carried out using a VP-ITC calorimeter (Microcal) at 30 °C. PA$_{3}$3 monomer (5 µM) and CMG2$^{35-225}$ (70 µM) were degassed at 30 °C under vacuum. Multiple injections of CMG2$^{35-225}$ were made into the PA$_{3}$3-containing measurement cell under continuous stirring. Data were analyzed using MicroCal Origin software.

**Fluorescence Equilibrium Stoichiometry Titrations**—PA$_{3}$3 E733C AF488 was diluted to 1 µM in 2 ml of universal buffer, 10 mM each Tris, MES, HEPES, and acetic acid, and 0.1 mg/ml bovine serum albumin, pH 8.0, that was supplemented with 1 mM MgCl$_2$. 100 µl aliquots were iteratively removed after the addition of sub-stoichiometric quantities of AF488–PA$_{63}$E733C, thereby continually increasing the ratio of CMG2–PA$_{3}$3 during the titration from 0.1 to 5.2. Each 100-µl aliquot was separately incubated for about 1–2 h at room temperature to ensure complete binding.

Similarly, (PA$_{63}$$_{7}$E733C–AF488 was diluted to 16 nM; however, 0.5 µM LF$_{54}$ was added to the reaction mixture to minimize nonspecific interactions with the PA-LP binding face. The molar ratio was varied as described for PA$_{3}$3 except the range spanned 0.3–25.

Each aliquot was then analyzed in an ISS fluorimeter interfaced to an Ar$^+$ laser. The 488-nm line was used for excitation of the donor fluorophore (AF488). Donor and acceptor emission were acquired at 520 and 570 (±10) nm, respectively. The apparent fluorescence resonance energy transfer (FRET) signal was defined by the ratio of the donor to acceptor fluorescence emission. Aliquots were diluted to 2 ml in universal buffer. Fluorescence counts were recorded for 10 s, and all aliquots in a given titration were measured in triplicate.

**Multi Laser Light Scattering—**Approximately 200–400 µg of protein was loaded in specific mixtures onto a Shodex KW-803 column at a flow rate of 0.5 ml min$^{-1}$ in 20 mM Tris–Cl, 200 mM NaCl, pH 8.5. The column was connected to a DAWN EOS 18-angle light scattering detector and an OPTILAB DSP interferometric refractometer (Wyatt Technology). Detectors 6–15 were used. A refractive index increment value (dn/dc) of 0.185 ml g$^{-1}$ was used. Detectors were normalized to compensate for slight differences in electronic gain using bovine serum albumin as an isotropic scatterer. Data were analyzed using ASTRA software.

**Kinetiksof PA$_{3}$3 Heptamerization—**The FRET-based kinetics assay was used to monitor the rate of heptamerization for PA$_{3}$3. A reaction mixture of fluorescently labeled nicked PA$_{3}$3 (50 µl each donor, AF488, and acceptor, AF546) in 20 mM Tris–Cl, 150 mM NaCl, 1 mM Ca$^{2+}$, 0.1 mg/ml bovine serum albumin, pH 8.5, was either preincubated without CMG2$^{35-225}$ or 1 µM CMG2$^{35-225}$ at 20 °C for 15 min. At time 0 each heptamerization reaction was initiated by the addition of 100 nM LF$_{54}$. Control experiments were performed in the absence of LF$_{54}$. Kinetic profiles were recorded using the ISS fluorimeter (excitation, 488 nm), where the emission at 520 and 570 (±10) nm reflected the increase in apparent FRET upon heptamer formation. Time points were taken automatically every 15 s for 7 × 103 s.

**Surface Plasmon Resonance Analysis—**All experiments were performed using the Biacore 2000 system sensor chips. For CM5 chips, the system was maintained at a constant flow rate of 5 µl min$^{-1}$ of HBS buffer (10 mM HEPES, pH 7.4, 150 mM NaCl) at 25 °C. Monomeric PA$_{3}$3 was covalently linked to the carboxylated dextran matrix. Monomeric PA$_{3}$3 was diluted to 2 µM in sodium acetate buffer, pH 4.5. The protein was injected onto the activated surface at a flow rate of 5 µl min$^{-1}$ until the desired base-line level of 2000–4000 response units was obtained (−5 min) and then blocked with ethanolamine.
Results

Expression and Purification of CMG2 VWA Domain—Recombinant CMG2 VWA domain (CMG2\textsubscript{35–225}) was fused to the carboxyl terminus of GST, and the resulting GST-CMG2 fusion protein was expressed in Escherichia coli. The protein was affinity-purified from bacterial extract on a glutathione-Sepharose column. The protein was cleaved on the column with thrombin, releasing the CMG2 VWA domain into the fluid phase in almost pure form. After an additional size-exclusion chromatography step, the CMG2 VWA domain was obtained in high purity as judged by SDS-PAGE (Fig. 1C).

Binding Studies of an Anthrax Toxin Receptor—We measured the stoichiometry of CMG2 VWA domain binding to PA\textsubscript{83} by three independent methods, isothermal titration calorimetry, FRET-based titration, and size-exclusion chromatography coupled with multiangle laser light scattering.

For isothermal titration calorimetry measurements, PA\textsubscript{83} was loaded into the measurement cell, and CMG2\textsubscript{35–225} was added in small increments under continuous stirring. The concentration of PA\textsubscript{83} used was 5 µM, orders of magnitude above the K\textsubscript{d} (see Table I), allowing incremental substoichiometric amounts of CMG2\textsubscript{35–225} to bind PA\textsubscript{83} quantitatively up to the point of saturation. As shown in Fig. 2, saturation by CMG2\textsubscript{35–225} was achieved at approximately a one-to-one molar ratio (1.03 \pm 5 \times 10^{-3}).

In a second approach we used FRET to report on the binding of CMG2 VWA domain and PA\textsubscript{83} under equilibrium conditions. A donor fluorophore (AF488) was attached to PA\textsubscript{83} via a unique, introduced cysteine (E733C), creating PA\textsubscript{83} E733C-AF488. A truncated version of CMG2, CMG2\textsubscript{240}, was modified on its amino terminus to have a single cysteine (CMG2\textsubscript{240}), which served as a unique site for fluorescent dye attachment. CMG2\textsubscript{240} was labeled by the acceptor fluorophore, AF546. The donor was excited by the 488-nm Ar+ laser line, and binding was reported by sensitized emission from the acceptor. The Förster distance, R\textsubscript{0}, for these two fluorophores is \(\sim 60\) Å; distances up to \(\sim 100\) Å generate enough signal to report binding. The degree of donor quenching indicated that the donor and acceptor in the PA-CMG2\textsubscript{240} complex are \(\sim 60–70\) Å apart (Fig. 3A). The concentration of PA used (1 µM), far above the K\textsubscript{d}, yielded a titration style plot (Fig. 3B).

Successive additions of the acceptor CMG2 increased the FRET signal until all PA\textsubscript{83} acceptor was bound, and no additional FRET was observed upon further additions. The intersection of two lines from separate linear fits to the sub-stoichiometric and saturation data defines a binding stoichiometry of 1.0 \pm 0.1.

Using size-exclusion chromatography coupled with multiangle laser light scattering, the measured molecular mass of the PA\textsubscript{83} CMG2\textsubscript{35–225} complex, isolated as a discrete peak, was
98 kDa and agrees with the theoretical value, 104 kDa, for a one-to-one complex. Molecular mass values were constant across the peak, indicating homogeneity of the complex.

**PA63 Heptamer Binds 7 Eq of CMG2**

We performed a FRET binding titration assay similar to that carried out on monomeric PA83 to determine the number of CMG2 VWA domains that bind uniformly labeled \((\text{PA}_63)^7\). Fluorescently labeled PA83 was treated with trypsin, and the PA63 fraction was isolated by anion-exchange chromatography. This fraction was shown earlier to be heptameric by light scattering (7) and X-ray crystallography (18). For fluorescence titrations, successive substoichiometric additions of the acceptor CMG2 increased the FRET signal until all \((\text{PA}_63)^7\) binding sites were saturated. Subsequent additions revealed no additional FRET (Fig. 3C).

The solution of the intersecting linear fits of data compiled from three separate titrations defines a binding stoichiometry of \(7.1 \pm 0.3\) CMG2 VWA domains per heptamer.

Mass estimates from size-exclusion chromatography coupled with multiangle laser light scattering showed that \((\text{PA}_63)^7\) binds at least 5 molecules of CMG2 but values of the masses were not sufficiently accurate to confirm the 7-fold stoichiometry offered by FRET. This approach suffered from the fact that the mass of each molecule of CMG2 (21 kDa) that bound to \((\text{PA}_63)^7\) (441 kDa) is equivalent to the measurement error for this method when working in the half-million dalton range (±5%).

**Does CMG2\(^{233-225}\) Affect the Rate of PA\(_{83}\) Heptamerization?**—We developed a second novel FRET method to monitor \((\text{PA}_83)^7\) prepore formation in solution to test whether CMG2\(^{233-225}\) affected the assembly process. In this assay donor (AF488)- and acceptor (AF546)-labeled PA83 were made by modifying a unique, solvent-accessible cysteine engineered at residue 563. Fluorescently labeled PA83 proteins were then nicked with trypsin. The heptamerization reaction mixture contained equimolar amounts of nicked donor and acceptor PA83 (50 nM each). At time 0, LF\(_N\) (the LF amino-terminal domain) was added (100 nM) to initiate the reaction. Control experiments verified that LF\(_N\) accelerated the heptamerization reaction.

The rate of heptamerization was reported by the increase in FRET due to the incorporation of donor and acceptor PA83 into the \((\text{PA}_83)^7\)-LF\(_N\) complex (Fig. 4). The kinetics fit to a second order rate expression with an observed rate constant of \(8.8 \times 10^{-8}\) s\(^{-1}\). We then compared the rate of heptamerization when the same equimolar mixture of nicked fluorescent PA83 was prebound by a 10-fold excess of CMG2\(^{233-225}\). The rate constant for \((\text{PA}_83)^7\) heptamerization, \(7.8 \times 10^{-4}\) s\(^{-1}\), was not significantly altered by the presence of CMG2\(^{233-225}\).

**Binding Kinetics of CMG2 to PA\(_{83}\)**—We used surface plasmon resonance (SPR) and FRET to obtain association and dissociation rate constants. For SPR studies, PA83 was bound to the dextran surface of a CM5 chip by amine coupling. Serial injections of CMG2\(^{233-225}\) showed that it bound to the immobilized PA83, and association kinetic traces were readily measured (Fig. 5A). Values of \(k_a\) and \(k_d\) measured with two different PA83 preparations and two different CM5 chips were similar.
Fig. 4. The kinetics of PA$_{83}$ heptamerization. For the FRET-based kinetics assay, a reaction mixture of fluorescent nicked PA$_{83}$ (50 nm each donor, AF488, and acceptor, AF546), 1 mM Ca$^{2+}$, 0.1 mg/ml bovine serum albumin, pH 8.5, was preincubated with either no CMG2$^{35-225}$ (fit with a solid line) or 1 µM CMG2$^{35-225}$ (fit with a short dashed line) at 20°C for 15 min. At time 0, each heptamerization reaction was accelerated by the addition of 100 nM LF$_N$. The control reaction, in the absence of LF$_N$ (fit by long dashed line), shows little increase in FRET. Kinetic profiles indicate an increase in FRET due to the incorporation of donor and acceptor PA$_{83}$ into the heptamer-LF$_N$ complex. Profiles fit to a second order rate expression (Equation 2) with rate constants of $8.8 \times 10^{-4}$ and $7.5 \times 10^{-4}$ s$^{-1}$ in the presence and absence of CMG2$^{35-225}$, respectively.

The average measured $k_a$ for CMG2$^{35-225}$ was $1.7 \times 10^5 \pm 7 \times 10^4$ M$^{-1}$ s$^{-1}$, with $R^2$ values greater than 0.999. The value of $k_a$ was $7 \times 10^{-5} \pm 5 \times 10^{-5}$ s$^{-1}$. The slow dissociation rate made accurate measurement difficult ($R^2 = 0.41$) because the base line drifted over time, and dissociation curve measurements (up to 150 s) had to be extrapolated to 0. From the association and dissociation rate constants, we calculated the equilibrium dissociation constant, $K_d$, to be $4 \times 10^{-5} \pm 2 \times 10^{-6}$ M.

Additional SPR measurements were made on CMG2$^{35-225}$, a truncated construct, lacking the natural disulfide bond between Cys-39 and Cys-218. This association rate, measured in the presence of Ca$^{2+}$, was found to be about 2-fold slower than CMG2$^{35-225}$ (Fig. 5C). Reduction of the disulfide bond in CMG2$^{35-225}$ similarly reduced the association rate by a factor of 2 with respect to the oxidized form (Table I).

Because of the inherent difficulties in measuring extremely slow dissociation rates (lifetimes $> 10^4$ s) using SPR, we developed a FRET-based binding system. Association rates were initially estimated using stopped-flow to confirm the fidelity of the novel VWA domain binding assay. Blue laser light was used to excite the donor fluorophore (AF488) on PA, which transferred energy to the acceptor fluorophore (AF546) on CMG2$^{340}$, allowing binding kinetics to be observed (Fig. 5B). Association rate constants ($k_a$) measured for this FRET-based system (1.1 $\times 10^5 \pm 5 \times 10^4$ M$^{-1}$ s$^{-1}$) were similar to those measured by SPR using CMG2$^{340}$ in the presence of Ca$^{2+}$ (Fig. 5C).

The dissociation rate was monitored using non-fluorescent CMG2$^{340}$-NEM (where the reactive cysteine was blocked by N-ethylmaleimide). Here, PA$_{83}$ E733C-AF488 and CMG2$^{340}$, AF546 were preincubated with 1 mM Ca$^{2+}$ buffer, and equivalent amounts were mixed to form a complex. The exchange reaction was initiated by adding a 20-fold excess of CMG2$^{340}$-NEM competitor. Kinetics, monitored over the course of $3 \times 10^4$ s, fit to a dissociation rate constant of $8.4 \times 10^{-6} \pm 5 \times 10^{-7}$ s$^{-1}$ (Fig. 5D). Thus, the $K_d$ is $7.8 \times 10^{-10} \pm 3 \times 10^{-11}$ M.

Taking advantage of this FRET system, we also measured the association rates of Mg$^{2+}$-bound CMG2, Here, protein solutions were preincubated with 2 mM MgCl$_2$ and 1 mM EGTA, reducing the effective concentration of even 1 µM contaminating Ca$^{2+}$ to less than 1 pM. Surprisingly, the association rate constant in the presence of Mg$^{2+}$, as compared with Ca$^{2+}$, is 2 times slower, at $5.3 \times 10^4 \pm 9 \times 10^3$ M$^{-1}$s$^{-1}$. Nonetheless, the dissociation rate was 10-fold slower ($9.2 \times 10^{-7} \pm 1 \times 10^{-7}$ s$^{-1}$; Fig. 5D), yielding a $K_d$ of $1.7 \times 10^{-10} \pm 9 \times 10^{-13}$ M.

Finally, we conducted binding kinetics experiments in metal ion “free” buffer that contains 2 mM EDTA and EGTA to chelate and sequester metal ions bound in the MIDAS motif and those contaminating the buffer solutions. The observed rate constants ($k_{obs}$), plotted in Fig. 5C, demonstrate a “non-linear” CMG2 concentration dependence (in the log-log plot), indicating that the [CMG2] used in the measurements is on the order of the $K_d$. The observed rate constant, $k_{obs}$, for binding is the sum of the association and dissociation rates,

$$k_{obs} = k_a + k_d \times [L]$$

(Eq. 3)

where only the association rate is dependent on the concentration of free ligand, [L]. The observed rates may then be fit by Equation 3 to obtain both the association and dissociation rate constants, $9.3 \times 10^6 \pm 7 \times 10^5$ M$^{-1}$s$^{-1}$ and $8.6 \times 10^3 \pm 4 \times 10^3$ s$^{-1}$, respectively. This model fit well, with an $R^2$ value of 0.85, and generates a $K_d$ of $9.6 \times 10^{-7} \pm 8 \times 10^{-8}$ M.

**Thermodynamic Contribution of the Metal Ion Site**—Because the affinity of CMG2 for PA could be measured in both the presence and absence of metal ions, the energetic contribution of the metal ion could be determined. This measurement is unique to our study of VWA domain binding, because often the removal of metal ions from the MIDAS completely abrogates binding. Knowing the affinity in the absence of metal ions allows the calculation to be made according to Equation 4,

$$\Delta G^{M^{2+}} = RT \ln \left( \frac{K_{d}^{M^{2+}}}{K_d} \right)$$

(Eq. 4)

in which the equilibrium dissociation constant in the absence ($K_d$) and presence of a metal ion ($K_{d}^{M^{2+}}$) are known. $R$ and $T$ are the gas constant and temperature, respectively. This model also assumes that the MIDAS is saturated, and the metal ion cannot freely exchange with the buffer, because it is buried in the binding interface. Estimates of $\Delta G^{M^{2+}}$ due to the presence of the bridging metal ion found in the MIDAS of CMG2 are $-5.1 \pm 0.05$ and $-4.2 \pm 0.05$ kcal mol$^{-1}$ for Mg$^{2+}$ and Ca$^{2+}$, respectively.

**DISCUSSION**

In the current study we have prepared soluble CMG2 VWA domain and quantitatively examined its interaction with monomeric and heptameric forms of PA. We studied the binding kinetics, the thermodynamics, and the role of metal ion in the interaction.

**Binding Stoichiometry to Monomeric and Oligomeric Forms of PA**—We quantified the number of CMG2$^{35-225}$ molecules bound to various forms of PA using three independent methods. All revealed that one CMG2$^{35-225}$ binds per PA$_{83}$ monomer. CMG2 binding stoichiometry for PA heptamer was measured using a FRET method identical to that used on PA$_{83}$ monomer. Titrations of labeled CMG2 C40 at concentrations 100-fold greater than the $K_d$ demonstrate that seven CMG2$^{340}$ moieties bind per heptamer. These results indicate that the heptamerized form of PA$_{83}$ does not preclude simultaneous occupancy of its seven individual CMG2 VWA domain binding sites, for example by steric obstruction.

Correspondingly, FRET studies on the heptamerization of PA$_{83}$ (Fig. 4) reveal that the rate of complex assembly in solution is not significantly different when PA monomer is liganded by CMG2$^{35-225}$. This result supports the FRET binding stoichiometry studies on heptamer; if the heptamerized form of PA could bind less than seven CMG2 VWA domains due to steric constraints, then the rate of assembly, $-10^{-3}$ s$^{-1}$, would be reduced, because CMG2 VWA domains would have to dissociate.
Table I

<table>
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<tr>
<th>Protein</th>
<th>Metal ion buffer</th>
<th>Method</th>
<th>$k_a$</th>
<th>$k_d$</th>
<th>$K_d$</th>
<th>$\Delta G^{M^{2+}}$</th>
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<tr>
<td>CMG225–225</td>
<td>1 mM Ca$^{2+}$</td>
<td>SPR</td>
<td>$1.7 \times 10^3 \pm 7 \times 10^4$</td>
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<td>SPR</td>
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<tr>
<td>Reduced S-S</td>
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<td>SPR</td>
<td>$8.1 \times 10^3 \pm 2 \times 10^4$</td>
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</tr>
<tr>
<td>CMG225–225-AF546</td>
<td>1 mM Ca$^{2+}$</td>
<td>FRET</td>
<td>$1.1 \times 10^7 \pm 5 \times 10^7$</td>
<td>$8.4 \times 10^{-5} \pm 5 \times 10^{-7}$</td>
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<td>CMG225–225-AF546</td>
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<td>FRET</td>
<td>$5.3 \times 10^4 \pm 9 \times 10^5$</td>
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* The equilibrium dissociation constant is calculated from kinetic measurements of the association and dissociation rate constants according to $K_d = k_d/k_a$.

* ND, not determined.

* Natural disulfide (Cys-39–Cys-218) is reduced by 0.5 mM tris-(2-carboxyethyl)phosphine.

at the slower rate, $10^{-5}$ s$^{-1}$, before assembly. In solution, the assembly of PA heptamer is not directly facilitated by the presence of CMG2 VWA domain. The modest 11% reduction in rate may reflect some minor steric or electrostatic repulsion from CMG2 VWA domain moieties in the assembly process.

Binding Affinity of CMG2 to PA—SPR measurements with CMG225–225 yielded $K_d$ values $\sim 400$ pM in the presence of Ca$^{2+}$. Stopped-flow FRET measurements on a truncated, disulfide-free version (CMG225–225) gave a $K_d$ of 780 pM in the presence of Ca$^{2+}$; however, when studied in the presence of a 1 mM effective concentration of Mg$^{2+}$, the $K_d$ became significantly tighter, 170 pM (Table I).

Our observation that the CMG2-PA binding interaction prefers Mg$^{2+}$ to Ca$^{2+}$ by 5-fold differs from a previous study (11). The enzyme-linked immunosorbent assay-based assay used in that study may have more closely sensed the 2-fold slower association rate for Mg$^{2+}$ with respect to Ca$^{2+}$ that we observed, thereby underestimating the affinity as Mg$^{2+}$-bound.
Dissociation rates vary from 10 s⁻¹ to 10 s⁻¹, and K_d values range from millimolar to submicromolar (20, 24–27). Affinity of CMG2, 170 μM, however, is 1000-fold greater in the absence of metal ions (28, 29), whereas we observed a 6000-fold reduction in affinity for PA–CMG2. NIF and PA may bind their receptor VWA domain in a metal ion-independent manner but to very different degrees, consistent with the model that a second MIDAS-independent binding site is utilized by CMG2. The tight binding strategy adopted by NIF clearly aims to block and inhibit adhesion-dependent responses of the neutrophil by competitively binding host integrin VWA domains (28).

The CMG2 role in toxin assembly and translocation—The high affinity and extremely slow dissociation rate of the PA-CMG2 VWA domain interaction, on the other hand, facilitates toxin assembly and translocation (Fig. 1A). For monomeric PA, the average lifetime of the dissociation, 1/k_d, for Mg²⁺-liganded CMG2 VWA domain is ~30 h. Thermodynamically, when considering the 7-fold stoichiometry of CMG2–liganded heptamer, the additive metal ion binding free energy ΔG_Mg²⁺ for seven independent PA-CMG2 interactions is 35 kcal mol⁻¹, favoring bound prepropeptide to free by a factor of 10²⁵.

During anthrax intoxication, PA must remain bound initially to the extracellular membrane surface and subsequently to the inner surface of a vesicle during the translocation process. An initial role of the receptor may be to increase the cell surface concentration of PA monomer, thereby promoting heptamer formation. Second, the receptor ensures that heptamer remains bound to the cell, allowing EF and LF to bind and assemble into a complex. Finally, tight affinity presumably permits anthrax toxin to assemble and translocate active LF.
and EF proteins in the very earliest stages of the intoxication, even when these proteins are low in concentration.

The CMG2 ~200 pM affinity for PA may also be exploited therapeutically as a potent anti-toxin. The tight binding and slow dissociation kinetics indicate that the CMG2 VWA domain may be delivered in soluble form to compete for the domain 4 binding site on monomeric PA$_{23356}$, blocking the PA ability to bind receptors, assemble, and translocate the enzymatic effectors, LF and EF, into target cells.

Acknowledgments—We thank L. Greene and R. Pimental for assistance in purifying proteins, J. A. T. Young, H. Scobie, and J. Rainey for the CMG2 clone and critical comments on the manuscript, and T. Sosnick for his critical reading of the manuscript.

REFERENCES