A Quantitative Study of the Interactions of *Bacillus anthracis* Edema Factor and Lethal Factor with Activated Protective Antigen†

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ABSTRACT: *Bacillus anthracis* secretes three proteins, which associate in binary combinations to form toxic complexes at the surface of mammalian cells. Receptor-bound protective antigen (PA) is proteolytically activated, yielding a 63 kDa fragment (PA_{63}). PA_{63} oligomerizes into heptamers, which bind edema factor (EF) or lethal factor (LF) to form the toxic complexes. We undertook a quantitative analysis of the interactions of EF with PA_{63} by means of surface plasmon resonance (SPR) measurements. Heptameric PA_{63} was covalently bound by amine coupling to an SPR chip, or noncovalently bound via a C-terminal hexahistidine tag on the protein to Ni^{2+}nitrilotriacetate groups on the chip. Values of k_{on} and k_{off} for EF at 23 °C were $\sim 3 \times 10^5$ M^{-1} s^{-1} and $(3-5) \times 10^{-4}$ s^{-1}, respectively, giving a calculated K_d of $(1-2) \times 10^{-9}$ M. A similar value of K_d (7×10^{-10} M) was obtained when we measured the binding of radiolabeled EF to receptor-bound PA_{63} on the surface of L6 cells (at 4 °C). Each of these analyses was also performed with LF and LF_N (the N-terminal 255 residues of LF), and values obtained were comparable to those for EF. The similarity in the dissociation constants determined by SPR and by measurements on the cell surface suggests that the presence of the receptor does not play a large role in the interaction between PA_{63} and EF/LF.

Certain *Bacillus* and *Clostridium* species produce an unusual category of AB toxins, termed binary toxins. Prototypic examples are the lethal and edema toxins of *Bacillus anthracis* (*1*). In these toxins, the A (enzymic) and B (receptor-binding) moieties are released from the bacteria as discrete, unassociated proteins, which come together and undergo a self-assembly process at the surface of receptor-bearing eukaryotic cells. The resulting complexes are then internalized, and the A moieties are delivered to the cytosol, where they catalyze reactions that elicit the toxic responses. For self-assembly at the cell surface to occur, the A and B moieties must have high affinities for one another.

The two anthrax toxins are assembled from three proteins; a single B protein serves as a delivery vehicle for two A proteins. Lethal toxin (LeTx)[1] is formed from protective antigen (PA; 83 kDa), the B protein, and lethal factor (LF; 90 kDa), the A protein (*1*). LF is a Zn^{2+}-dependent protease, which elicits hyperproduction of interleukin-1β and tumor necrosis factor-α by macrophages (*2*−*4*), causing potentially lethal shock. Edema toxin (EdTx) is assembled from edema factor (EF; 89 kDa), a calmodulin-dependent adenylyl cyclase, and PA (*5*). The target cells of EdTx are not known, but the elevation of cAMP concentrations in professional phagocytes by toxins affects cytokinesis and is believed to protect the producing bacteria from phagocytosis (*6*).

PA binds to a ubiquitous, but unidentified, surface receptor of 80−90 kDa (*7*) and is cleaved by furin or a furin-like protease at an Arg-Lys-Lys-Arg sequence (residues 164−167) (*8*). This generates an N-terminal 20 kDa fragment (PA_{20}) which dissociates, leaving the complementary C-terminal 63 kDa fragment (PA_{63}) bound to the receptor. Removal of PA_{20} allows PA_{63} to manifest two functional properties that are absent from native PA: (i) PA_{63} binds EF or LF, noncovalently and competitively (*9*, *10*); and (ii) it spontaneously self-associates, forming seven-membered ring-shaped oligomers (*11*, *12*). The temporal order of the heptamerization and EF/LF binding events remains unclear.

After self-assembly, the toxin complexes are internalized and delivered to an acidic compartment (*13*). There, acidification leads to translocation of EF and LF to the cytosol (*14*). The mechanism by which these proteins cross the endosomal membrane remains elusive, but low pH causes insertion of heptameric PA_{63} into membranes, creating a transmembrane channel, which may serve as the pathway of translocation (*12*, *15*, *16*). Recent studies indicate that heptameric PA_{63} can exist in two states: a prepore (pore precursor), which is stable in solution at pH >8 and dissociates in SDS, and a pore, which forms under acidic conditions and is stable in SDS. The prepore-to-pore conversion involves a major conformational change which includes formation of a transmembrane 14-stranded β-barrel (*15*, *17*).

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[1] Abbreviations: AT, anthrax toxin; DMEM, Dulbecco's modified Eagle's medium; EDC, *N*-ethyl-*N*′-[(3-dimethylamino)propyl]carbodiimide hydrochloride; EdTx, edema toxin; EF, edema factor; HBS, HEPES-buffered saline; HBSE, HEPES-buffered saline, pH 8.0, containing EDTA; LeTx, lethal toxin; LF, lethal factor; LF_N, N-terminal 255 residues of LF; MES, 4-morpholineethanesulfonic acid; NHS, *N*-hydroxysuccinimide; nPA, trypsin-nicked protective antigen; NTA, nitrilotriacetic acid; PA, protective antigen; PA_{20}, N-terminal 20 kDa fragment of PA; PA_{63}, C-terminal 63 kDa fragment of PA; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; RU, reflectance unit(s); SPR, surface plasmon resonance.

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In the current study, we used surface plasmon resonance technology to examine the interaction of purified PA_{63} with its ligands in real time. The binding and off-rate constants determined in a model biophysical system were then compared with values for these constants determined by measuring the interaction of radiolabeled EF or LF with PA_{63} on the surface of L6 cells in culture.

EXPERIMENTAL PROCEDURES

Cell Culture. Rat myoblast cells (L6) were obtained from the American Type Culture Collection (ATCC CRL-1458). Cells were maintained in DMEM supplemented with 10% calf serum, 500 units/mL penicillin G, and 500 units/mL streptomycin sulfate (Gibco Life Technologies).

Preparation of Proteins. PA, LF, and EF were purified from the *B. anthracis* Sterne strain as described (*18*, *19*). Recombinant forms were purified from *E. coli* bearing the appropriate plasmid construct: pET15b (EF, LF) or pET22b (PA) (*15*) (Novagen). The pET15b construct adds a hexahistidine tag to the N-terminus of the proteins, permitting their purification by nickel chelate affinity chromatography. Purification of EF, LF, LF_N, PA_{20}, and EF_{62} was performed as described (*20*). The pET22b construct appends the pelB leader sequence to the N-terminus of PA, directing its expression to the periplasm. PA was purified from periplasmic extracts by manufacturer instructions (Novagen) and published procedures (*21*). Further purification of all proteins followed, using Fast Performance Liquid Chromatography (Pharmacia) Q-Sepharose and Mono Q anion exchange columns (PA), or just Mono Q alone (EF, LF, LF_N, PA_{20}, EF_{62}) with 20 mM Tris, pH 8.0, as buffer A and A + 1 M NaCl as buffer B.

Iodination of EF and LF. Purified EF was buffer-exchanged and concentrated using Centricon-30 filter membrane units (Amicon). EF was exchanged into 0.1 M sodium phosphate, pH 8.0, buffer, while LF was exchanged into this same buffer also containing 10 μM $CaCl_2$, 10 μM $ZnSO_4$, and 10 μM $MgCl_2$. One millicurie of Bolton−Hunter reagent (NEN-Dupont) was divided into two parts, each of which was dried at 4 °C under a stream of nitrogen. Thirty micrograms of protein was added to the dried Bolton−Hunter reagent (15 μL total volume), and the mixture was incubated for 90 min at 0 °C with gentle agitation for the first 15 min. The reactions were stopped by the addition of 0.2 M glycine, 0.1 M sodium phosphate, pH 8.0, to 500 μL and continued incubation at 0 °C for 5 min. Samples were freed from unreacted Bolton−Hunter reagent by NAP-5 (Pharmacia) gel filtration columns equilibrated in 20 mM Tris, pH 8.0, 0.1% gelatin (a carrier). One milliliter fractions were collected and subjected to gamma radiation analysis and gel electrophoresis. The concentration of labeled EF or LF was measured by micro-BCA assay (Pierce). Autoradiography of LF gel samples demonstrated that LF was substantially contaminated by breakdown products after the iodination procedure; thus, subsequent experiments with this protein relied on gel electrophoresis followed by phosphorimagery for quantitative analysis.

Cell Binding Assay for K_d Determination. L6 cells were plated at a density of 2×10^5 cells/well in 24-well culture plates (Falcon). After 16 h, the medium was aspirated and replaced with 250 μL of buffered DMEM (10 mM HEPES) per well, containing either 0 or 2×10^{-8} M purified oligomeric PA_{63} at 4 °C. Plates were incubated at 4 °C for 2 h. The medium was removed by aspiration, and the cells were washed gently 2 times with cold phosphate-buffered saline, pH 7.5 (PBS). Buffered DMEM (250 μL) containing various concentrations of ^{125}I-labeled LF or EF (350 pM to 15 nM) was added to each well. Plates were incubated at 4 °C for 2 h. The medium was aspirated; the monolayers were washed twice with PBS to remove unbound protein, and then aspirated to dryness. Cells were solubilized with lysis buffer (0.1 M NaCl, 0.02 M NaH_2PO_4, 0.01 M EDTA, 1% Triton X-100) and transferred to individual tubes. The radioactive content of EF was measured by a gamma counter (LKB Wallac CliniGamma 1272). LF and its degradation products were precipitated from the lysate by the addition of 5% TCA, separated by SDS−PAGE on 12.5% gels, and analyzed by phosphorimagery. Collected data were subjected to Scatchard analysis as well as nonlinear regression, using the software package Prism 2.0 (Graphpad).

Cell Binding Assay for k_{off} Determination. This assay was similar to the K_d determination assay described above, except that each well contained the same concentration of labeled protein. After removal of the labeled protein medium by aspiration and washing of the cells twice with PBS, buffered DMEM containing 5×10^{-7} M unlabeled LF or EF was added to the cells, 250 μL/well. Plates were kept on ice, and at specified time points, the medium was collected and cells were solubilized with lysis buffer. The radioactive content of EF samples was measured by a gamma counter (LKB Wallac). For LF samples, proteins from each fraction were precipitated. The proteins in the medium were precipitated by the addition of acetone to 80% followed by overnight incubation at −20 °C; the cellular proteins were precipitated by the addition of TCA to 5% followed by a 30 min incubation on ice. Cellular protein pellets were washed twice (once for an hour, once overnight) with ethyl ether (Aldrich) at 4 °C with rotation. Pellets were air-dried and then heated at 95 °C for 5 min in sample buffer prior to SDS gel electrophoresis. Gels were fixed, dried under vacuum, and examined by phosphorimagery using a BI phosphor screen (BioRad). Relevant protein bands were quantified.

The experiment shown in Figure 6 was performed similarly to those described above, except that trypsin-nicked PA was bound to cells for approximately 2 h and then exposed to MES/gluconate buffer (140 mM NaCl, 5 mM sodium gluconate, and 20 mM MES) at pH 4.8 or 8.2 for 5 min. LF_N that had been synthesized and labeled with [^{35}S]-methionine as per manufacturer instruction (Promega, Madison, WI), and dialyzed against MES/gluconate, pH 8.2, overnight to remove unreacted [^{35}S]methionine, was added to the cells for 2 h. The cells were then washed twice with PBS and exposed to MES/gluconate, pH 8.2, containing 2×10^{-7} M unlabeled LF_N. Buffer was removed at various times and measured for radioactivity.

Surface Plasmon Resonance Analysis. All experiments were performed using the BIAcore 2000 system sensor chips and software evaluation package (Pharmacia Biosensor, Uppsala, Sweden). For CM5 chips, the system was maintained in a constant flow of 10 μL/min HBS buffer (10 mM HEPES, pH 7.4, 150 mM NaCl) at 23 °C. Oligomeric PA_{63} was covalently bound to the carboxylated dextran matrix by amine coupling following manufacturer directions (Pharma-

cia) with the following exceptions: the oligomer was diluted into sodium acetate buffer, pH 5.5, to a concentration of between 200 and 800 nM. This was injected onto the activated surface at a flow rate of 2 μL/min until the desired baseline level was reached (1000−3000 RU) (less than 10 min), and then blocked with ethanolamine according to manufacturer instructions.

For NTA chips, the running buffer consisted of 10 mM HEPES, pH 8.0, 150 mM NaCl, 50 μM EDTA (HBSE). The methods used at all temperatures were identical, with the exception that the running buffer was degassed daily when the system was maintained above room temperature. The NTA-dextran surface was first stripped of any residual Ni^{2+} ions with 0.35 M EDTA, and subsequently charged with a 30 μL pulse of 100 μM $NiSO_4$, at a flow of 10 μL/min. A characteristic baseline increase of 50−60 RU was detected after this injection. Purified oligomer with a C-terminal hexahistidine tag was diluted into running buffer to a final concentration of 100 nM, and injected at a flow rate of 2 μL/min until the desired baseline was attained (<10 min, final baseline 700−6000 RU).

On both surfaces, serial injections of EF or LF diluted into running buffer (10−300 nM) were made using kinject with a 20 μL (amine) or 40 μL (NTA) pulse followed by a 150 s dissociation time. CM5 oligomer baselines were regenerated with a 10 μL pulse of 0.5 M $NaCO_3$, pH 10.5, which resulted in <3% loss of the baseline/injection. NTA oligomer baselines were regenerated by a 10 μL pulse of 2 M $MgCl_2$, with <5% loss of baseline observed per injection. The original NTA surface was regenerated by 2 μL/min injection of 0.01 mg/mL trypsin in running buffer, followed by a 30 μL injection of regeneration solution (10 mM HEPES, 0.15 M NaCl, 0.35 M EDTA, pH 8.3) to strip nickel and remaining bound hexahistidine tags from the surface.

The association and dissociation rate constants k_{on} and k_{off} were determined from sensorgram data using the BiaEvaluation 2.1 software package.

RESULTS

SPR Analysis with Amine-Coupled PA_{63}. PA_{63} was purified from trypsin-nicked PA at pH 8.5 by anion exchange chromatography. PA_{63} purified by this method is believed to be all in a heptameric state, although the presence of monomers or lower order oligomers has not been excluded. PA_{63} was bound to the carboxymethylated dextran backbone of a CM5 chip by primary amine coupling to give a baseline increase of at least 2000 RU. The preconcentration step of this coupling requires lowering the pH of the protein to be immobilized to a value below its pK_a, calculated to be 5.7 for PA_{63}. The highest pH at which this preconcentration step was effective was pH 5.5. The interaction between immobilized PA_{63} and various analytes was examined at 23 °C (Figure 1A,B). Analyte diluted into HBS was injected over the PA_{63} surface at 10 μL min^{-1} for 120 s, allowing association to take place. Dissociation was then monitored in a constant flow of HBS for at least 150 s. Bound analyte was removed and the PA_{63} baseline regenerated with a 10 μL pulse of 0.5 M Na_2CO_3, pH 10.5. Baseline decay was <3% per cycle.

Serial injections of EF or LF at various concentrations (Figure 1A,B) showed strong binding of both proteins to

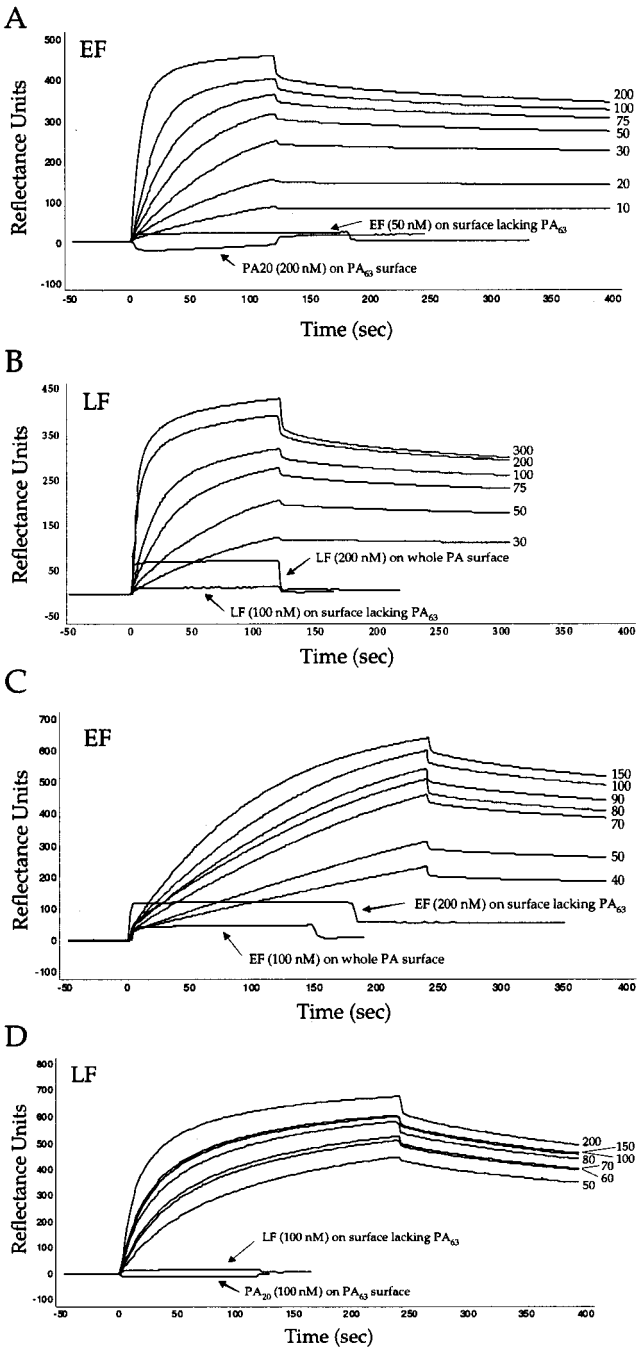

FIGURE 1: SPR analysis of binding of EF (panels A and C) or LF (panels B and D) to PA63 immobilized to a dextran matrix flow cell surface by primary amine coupling (panels A and B) or by a C-terminal hexahistidine tag:NTA-dextran interaction (panels C and D). Injections of EF or LF (indicated concentrations are in nM) in HBS were made over the PA63 surfaces, and the binding interactions were recorded in real time. Injections were at 10 μL/min for 120 s (panels A and B) or for 240 s (panels C and D), followed by passage of HBS buffer alone. Various controls were as indicated.

PA_{63}. LF_N, the N-terminal PA-binding domain of LF, also bound tightly (data not shown), whereas no binding was detected with PA_{20}, as expected (Figure 1A). EF_{62}, a fragment of EF lacking the N-terminal PA-binding domain, interacted with residual carboxyl groups on dextran on the surface of the flow cell, but showed no specific binding to the oligomer (data not shown). EF, LF, and LF_N did not bind to immobilized whole PA (Figure 1B) or to a protein-free surface blocked with ethanolamine (Figure 1A,B).

Table 1: Binding Constants for the Interaction of PA_{63} with EF and LF on Cells and As Determined by SPR

		n[a]	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})	k_{off} χ^2	K_d (M)
amine-coupled PA_{63}, 23 °C	EF	12	$2.6 \times 10^5 \pm 8.3 \times 10^3$	$3.4 \times 10^{-4} \pm 1.5 \times 10^{-4}$	0.032	$1.3 \times 10^{-9} \pm 5.8 \times 10^{-10}$
	LF	9	$2.1 \times 10^5 \pm 7.3 \times 10^3$	$5.9 \times 10^{-4} \pm 1.8 \times 10^{-4}$	0.032	$2.8 \times 10^{-9} \pm 8.4 \times 10^{-10}$
NTA-coupled PA_{63}, 23 °C	EF	10	$3.0 \times 10^5 \pm 7.1 \times 10^3$	$5.4 \times 10^{-4} \pm 4.7 \times 10^{-5}$	0.056	$1.8 \times 10^{-9} \pm 1.6 \times 10^{-10}$
	LF	7	$1.4 \times 10^5 \pm 3.1 \times 10^3$	$1.6 \times 10^{-3} \pm 8.6 \times 10^{-5}$	0.018	$1.1 \times 10^{-8} \pm 6.7 \times 10^{-10}$
L6 cells, 4 °C	EF			1.4×10^{-4}		7.0×10^{-10}
	LF			5.2×10^{-4}		4.0×10^{-10}

[a] Number of data sets involved in the determination of the constants.

Values of k_{on} for EF and LF were measured from data during the association phase, where ln $|dY/dX|$ was most linear (generally 50−100 s postinjection). Values of k_{off} were measured from 50 s of data during the dissociation phase, beginning 12 s after the end of injection. The data were fitted with the BiaEval analysis program to association and dissociation models in which the analyte binds to one or to more than one site on the immobilized PA_{63}. As judged by χ^2 values (Table 1), residual plots, and offset values, the data fit best to an association model in which EF or LF binds to one class of sites on the immobilized PA_{63}, and a dissociation model in which two different analyte−ligand complexes dissociate simultaneously. The slower of the two off rates calculated by this model (10^{-3}−10^{-4} s^{-1}) corresponds to ~97% of the amplitude (300−1000 RU) and thus presumably reflects interactions of the native proteins. The faster off rate (0.1−0.05 s^{-1}) corresponds to less than 3% of the total amplitude (<10 RU) and may reflect dissociation of complexes involving aberrant forms of PA_{63}, or EF and LF. The on rate constants measured for EF and LF were similar: 2.6×10^5 and 2.1×10^5 M^{-1} s^{-1}, respectively; and the corresponding off rate constants, 3.4×10^{-4} and 5.9×10^{-4} s^{-1}, differed from each other by less than a factor of 2. The equilibrium constants, K_d, calculated from these kinetic constants are 1.3×10^{-9} M for EF and 2.8×10^{-9} M for LF.

SPR Analysis with PA_{63} Bound via a Hexahistidine Affinity Tag. Amine coupling to the dextran matrix may occur via Lys residues exposed at various locations on the surface of a protein. Presumably, this method of ligand immobilization yielded a heterogeneous population of bound PA_{63} heptamers, in which a fraction of the sites for EF or LF may have been perturbed. Also, the exposure of PA_{63} to acidic conditions during the preconcentration step of amine coupling would be expected to convert the prepore to the pore form of the heptamer. Therefore, we performed additional studies using a different method of coupling, which linked PA_{63} to the matrix via a specific site on the protein and avoided acidic conditions.

We used a Ni^{2+}-complexed, NTA-derivatized chip, designed to tether hexahistidine-tagged proteins (Pharmacia) (*22*, *23*), and bound to it a form of PA_{63} bearing a hexahistidine tag fused to the C-terminal receptor-binding domain. From the crystallographic structure of the prepore (*12*), the seven tags on the heptamer would be expected to lie on the face opposite that of the putative EF/LF binding site. Thus, heptamer bound to NTA groups at the surface of the flow cell should have its EF/LF binding sites fully accessible to analyte. Also, because the pH was maintained at ≥8.0, we assume that the heptamer remained in the prepore form.

PA_{63}−H_6 was purified at pH 8.5 and tethered to the NTA chip in pH 8.0 buffer. Purified heptamer (100 nM) was applied in HBS containing 50 μM EDTA, pH 8.0, (HBSE) at 2 μL/min until a signal of 800−2000 RU was reached; then HBSE was passed over this surface (10 μL/min) until the baseline stabilized. A cycle consisted of an injection of analyte in HBSE at 10 μL/min for 4 min, followed by a dissociation period of 125 s. Regeneration of this surface with 10 μL of 2 M $MgCl_2$ caused less than 5% decay of the PA_{63} baseline.

Both EF and LF interacted avidly with the PA_{63}-containing surface, but not with the protein-free surface or with bound native PA (Figure 1C,D). LF_N showed about the same affinity for the heptamer as EF and LF did (data not shown), and PA_{20} did not bind at all (Figure 1D). The heptamer was difficult to remove from the surface; EDTA had little effect, and trypsinization or SDS treatment was necessary to strip the surface. This is consistent with the notion that each heptamer bound to the flow cell surface via multiple hexahistidine tags. The kinetic constants determined by this method were similar to those determined from the amine-bound PA_{63} (Figure 1C,D; Table 1). For EF, the value for k_{on} was 3.0×10^5 M^{-1} s^{-1} and for k_{off}, 5.4×10^{-4} s^{-1}. LF gave values of 1.4×10^5 M^{-1} s^{-1} for k_{on} and 1.6×10^{-3} s^{-1} for k_{off}. From these results, we calculated K_d values of 1.8×10^{-9} M for EF and 1.1×10^{-8} M for LF.

Effect of Temperature on Kinetic Constants. A major benefit of the BIAcore system is the ease with which effects of environmental perturbations on binding parameters may be measured. We examined the role of temperature on the binding of EF to the hexahistidine-tagged prepore immobilized on the NTA surface (Table 2). Values of k_{on} were similar at all temperatures examined, whereas k_{off} decreased by about an order of magnitude as the temperature was lowered from 30 °C ($k_{off} = 2.1 \times 10^{-3}$ s^{-1}) to 4 °C ($k_{off} = 1.9 \times 10^{-4}$ s^{-1}). From these data, we calculated the activation energy (E_a) by Arrhenius plots for the dissociation (13 kcal/mol) of the EF−PA_{63} complex (Figure 2). Thus, there is a low energy barrier to the formation of the complex and a larger one to its dissociation.

Effect of Acidic pH on the LF−PA_{63} Interaction. The fact that acidification induces translocation of EF and LF to the cytosol implies that these PA_{63} ligands are released from the oligomer under acidic conditions. It should be noted, however, that an acid-triggered release has not yet been demonstrated. To determine whether release from PA_{63} could be detected in response to low pH, LF was injected at neutral pH over the amine-coupled PA_{63} surface, and this was immediately followed by an injection of protein-free 10 mM sodium acetate buffer, pH 5.2. As shown in Figure 3, LF did not dissociate from PA_{63} more rapidly in this buffer than

Table 2: Binding Constants for the EF−PA_{63} Interaction on the NTA-Dextran BIAcore Surface at Different Temperatures

temp (°C)	n[a]	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})	$k_{off}\chi^2$	K_d (M)
4	12	$2.8 \times 10^5 \pm 5.6 \times 10^3$	$1.9 \times 10^{-4} \pm 5.1 \times 10^{-5}$	0.008	$6.8 \times 10^{-10} \pm 1.8 \times 10^{-10}$
15	5	$4.5 \times 10^5 \pm 1.1 \times 10^4$	$6.3 \times 10^{-4} \pm 1.5 \times 10^{-5}$	0.006	$1.4 \times 10^{-9} \pm 4.8 \times 10^{-11}$
23	10	$3.0 \times 10^5 \pm 7.1 \times 10^3$	$5.4 \times 10^{-4} \pm 4.7 \times 10^{-5}$	0.056	$1.8 \times 10^{-9} \pm 1.6 \times 10^{-10}$
30	11	$5.1 \times 10^5 \pm 1.7 \times 10^4$	$2.1 \times 10^{-3} \pm 1.6 \times 10^{-4}$	0.016	$4.1 \times 10^{-9} \pm 3.5 \times 10^{-10}$

[a] Number of data sets involved in the determination of the constants.

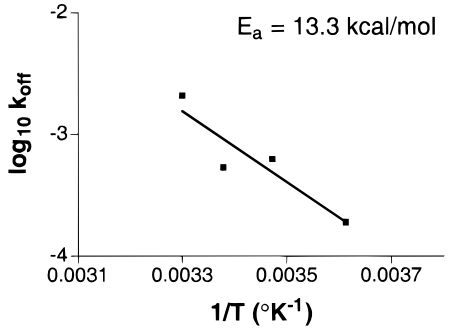

FIGURE 2: Activation energy for k_{off} of EF for PA_{63}. Measurements of k_{off} for EF binding to PA_{63} immobilized to the NTA-dextran surface in the BIAcore at 3.8, 15, 23, or 30 °C (Table 2) were subjected to analysis by Arrhenius plot.

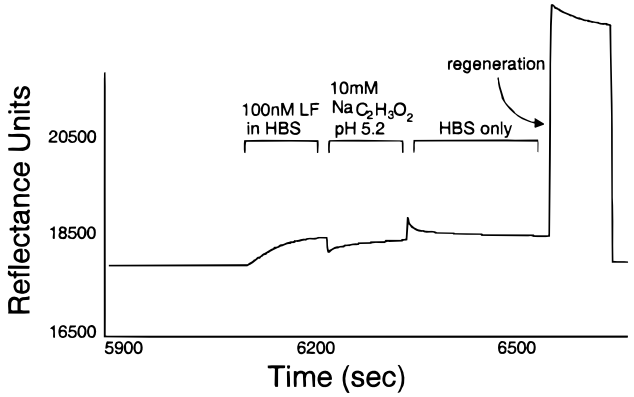

FIGURE 3: Effect of acidification on the LF−PA_{63} interaction. An injection of LF (100 nM) was made at 23 °C over a surface of PA_{63} amine-coupled to dextran. This 150 s injection was immediately followed without interruption by a pulse of 10 mM sodium acetate, pH 5.2, containing no LF, for 150 s. At the end of this pulse, the surface was returned to a constant flow of the standard buffer, HBS. No dissociation of LF from the surface was observed during the acetate pulse; the dip in the curve is due to the different refractive index of the buffer. Once returned to HBS, the off rate was measured to be 1.2×10^{-4} s^{-1}, roughly 5-fold slower than without acidification.

at neutral pH. The k_{off} of the LF remaining bound to PA_{63} throughout this low pH pulse was 1.2×10^{-4} s^{-1}, similar to that without acidification. After the pH was returned to 7.4, the off rate was found to be 5-fold slower than without acidification.

Measurements of K_d at the Surface of L6 Cells. To determine whether our findings from SPR studies were an accurate representation of interactions under physiological conditions, we determined equilibrium dissociation constants for the interaction of EF or LF with receptor-associated PA_{63} in cell culture. L6 cells were incubated with purified PA_{63} at pH 7.5 and were maintained at 4 °C to prevent endocytosis. After 2 h, unbound heptamer was removed by washing with cold PBS, and the monolayers were incubated with varying concentrations of ^{125}I-labeled LF or EF at 4 °C for an additional 2 h. The cells were then washed, solubilized, and examined for radioactive content. For EF, this consisted of measurements in a gamma counter (LKB-Wallac). Because of partial degradation of LF during the iodination process, these samples were precipitated with TCA, separated by SDS gel electrophoresis, and examined by phosphorimager for radioactive content. After correction for nonspecific binding using control cells lacking PA_{63}, the data were analyzed both by the method of Scatchard and by nonlinear analysis (Figure 4). The values determined by both methods were in good accord. For LF, the K_d was 4×10^{-10} M, and for EF, it was 7×10^{-10} M.

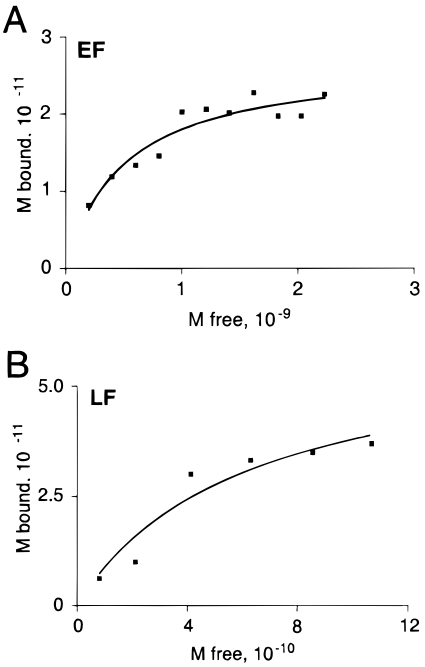

FIGURE 4: Binding of ^{125}I-LF or ^{125}I-EF to receptor-bound PA_{63}. L6 cells in culture were incubated with saturating amounts of purified PA_{63} for 2 h at 4 °C. Cells were washed with 4 °C PBS, and incubated with various concentrations of ^{125}I-EF (panel A) or ^{125}I-LF (panel B) at 4 °C for 2 h. The cells were again washed with cold PBS, solubilized in lysis buffer, and examined for radioactive content either by gamma counting (EF) or by TCA precipitation, SDS−PAGE, and phosphorimagery (LF). Nonspecific binding of labeled protein to L6 cells in the absence of PA_{63} (<5% of total binding) was subtracted from experimental measurements to obtain specific binding. Data were analyzed by nonlinear analysis and by the method of Scatchard.

These values are ~5-fold lower than those measured by SPR, but the measurements in these two systems were performed at different temperatures. We therefore determined the off rate of EF from cell-surface associated PA_{63} at 4 °C for comparison with SPR values obtained at this temperature. This assay involved a modified version of that for the K_d determination, in which all wells received the same concentration of iodinated protein. The cells were then washed and incubated with buffered medium containing an excess of

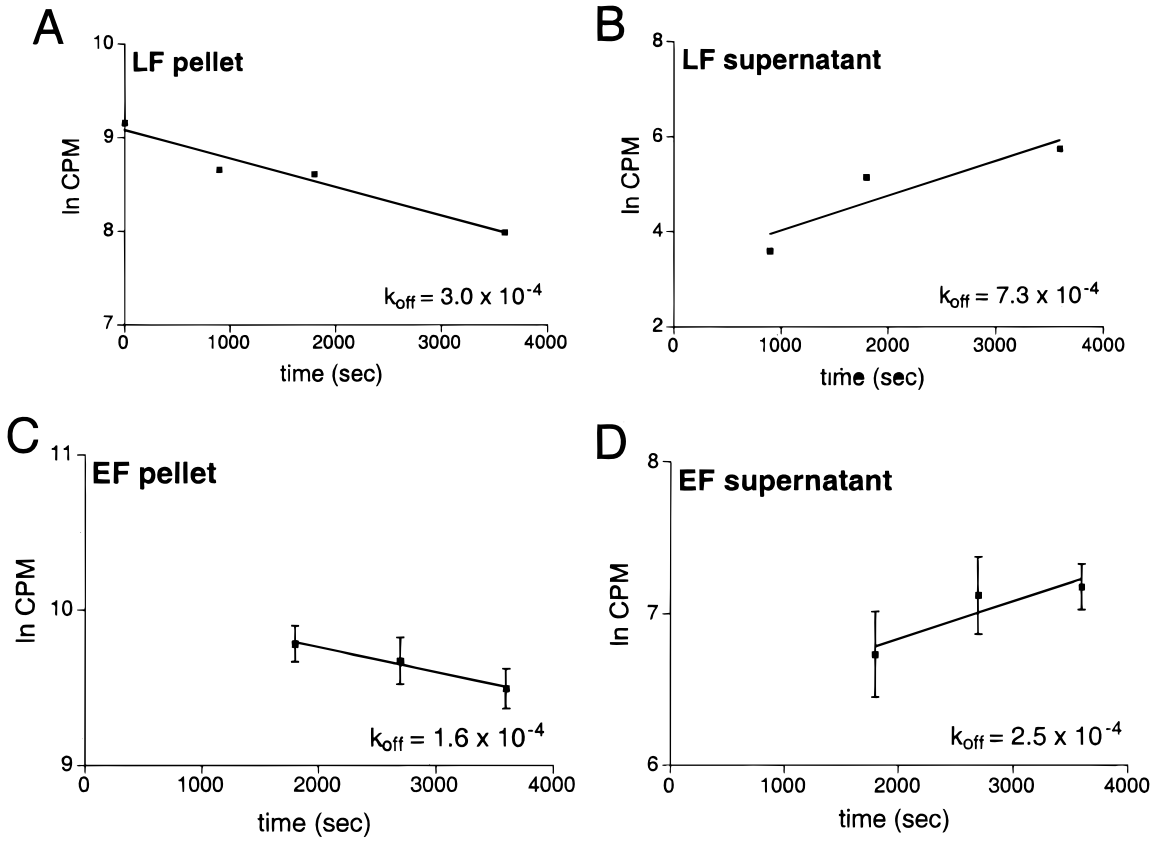

FIGURE 5: Determination of k_{off} for ^{125}I-LF or ^{125}I-EF bound to receptor-associated PA_{63}. L6 cells in culture were incubated with saturating amounts of purified PA_{63} for 2 h. Cells and buffers were kept at 4 °C throughout the experiments to prevent endocytosis. Cells were washed with PBS, and incubated with increasing concentrations of ^{125}I-LF (panels A and B) or ^{125}I-EF (panels C and D) at 4 °C for 2 h. The cells were again washed with PBS, and a 30-fold excess of unlabeled EF or LF was added. At specified time intervals, the medium from replicate wells of cells was collected, and the pellets were solubilized in lysis buffer. Both were examined for radioactive content either by gamma counter (EF) or by TCA precipitation, SDS−PAGE, and phosphorimagery (LF). Nonspecific binding (<20% of total for supernatant measurements; <5% of total for pellet measurements) from cells not treated with PA_{63} was subtracted from the measurements.

unlabeled LF_N to prevent rebinding of dissociated ^{125}I-protein. At various times, the medium was collected, the cells were solubilized, and the radioactive content of each sample was determined. Label present in the medium increased over time, and that associated with the cells decreased accordingly (Figure 5). Off rate constants estimated from the data—5.6 $\times$ 10^{-4} and 2.0 $\times$ 10^{-4} s^{-1} for LF and EF, respectively—are in good accord with the k_{off} of EF determined at 4 °C by SPR (1.9 $\times$ 10^{-4} s^{-1}). Thus, correcting for the change in k_{off} at the lower temperature, one obtains values for K_d on cells at the higher temperature that are within a factor of 2 of those obtained by SPR analysis.

We next examined whether the off rate of LF_N from prepore differed from the off rate of LF_N from pore. Trypsin-nicked PA was bound to cells at 4 °C for approximately 2 h. The heptamers, which form rapidly on the cell surface, were then exposed for 5 min to MES/gluconate buffer at pH 4.8 (which converted about 25% of the oligomers into the pore form; data not shown) or pH 8.2 (to maintain the prepore form). The cells were incubated with ^{35}S-labeled LF_N for 2 h, washed twice with PBS, and then incubated with excess unlabeled LF_N. At various times, the medium was collected and measured for radioactivity (Figure 6). The estimated off rates of LF_N from oligomers exposed to buffer at pH 8.2 (4.4 $\times$ 10^{-4} s^{-1}) and pH 4.8 (5.0 $\times$ 10^{-4} s^{-1}) are similar.

DISCUSSION

An essential step in the intoxication of cells by binary toxins is the process of self-assembly, in which the discrete A and B proteins associate to form functional complexes at the surface of the target cell. Leppla and co-workers were the first to show that proteolytic activation of PA by trypsin endows one of the resulting fragments, PA_{63}, with two new properties: (i) the ability to bind EF and LF, and (ii) the ability to self-associate to form a high M_r complex (*9*, *24*). While much has been learned in recent years about the structure and function of the anthrax toxin complexes, important questions have remained unresolved, including the binding constants of the interactions between PA_{63} and its ligands, EF and LF.

Estimates of the affinity of LF for PA vary widely in the literature. Novak et al. reported a K_d value of 2 $\times$ 10^{-10} M obtained by Scatchard analysis of data from an experiment involving binding of LF to nicked PA on J774A.1 cells in culture at 4 °C (*24*). The same group also reported in a review article a value of 1 $\times$ 10^{-11} M, however (*9*). The latter value was determined by an immunoprecipitation assay in which competition between radiolabeled LF and unlabeled LF and EF for binding to PA_{63} was measured (S. Leppla, personal communication). Last, LF was demonstrated to block the ion conductance of preformed PA_{63} membrane channels on

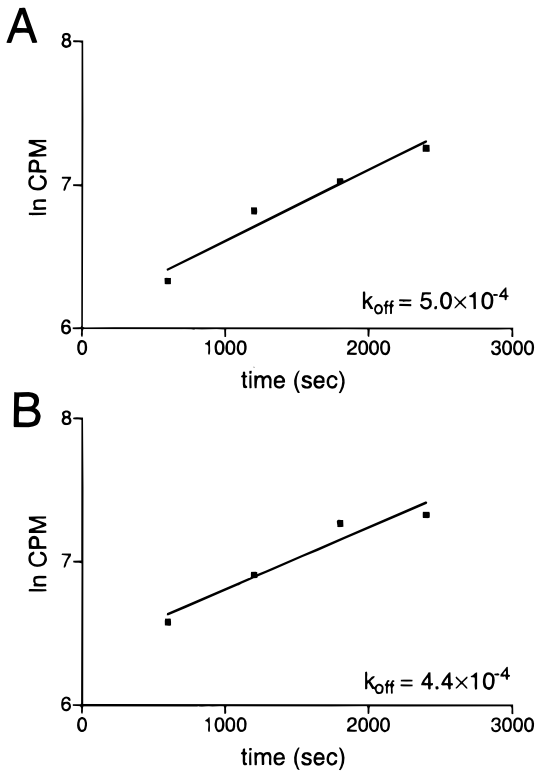

FIGURE 6: Determination of the k_{off} for ^{35}S-LF_N bound to prepore or pore forms of receptor-associated PA_{63}. L6 cells in culture were incubated with saturating amounts of purified trypsin-nicked PA for 2 h. Cells and buffers were kept at 4 °C throughout the experiments to prevent endocytosis. The cells were washed with PBS, and then exposed for 5 min to MES/gluconate buffer at pH 4.8 (panel A; to induce pore formation) or at pH 8.2 (panel B; to maintain the prepore form). Cells were then washed with PBS and exposed to ^{35}S-LF_N for 2 h. Cells were washed again with PBS and incubated with excess unlabeled LF_N, and then medium was removed at various times for measurement for radioactivity. Nonspecific binding (<10% of total) from cells not treated with PA_{63} was subtracted from the measurements.

the surface of CHO-K1 cells at 4 °C with half-maximal inhibition at 4×10^{-8} M (*20*). Given the large range of these reported values (4000-fold)—and in view of our increased understanding about the structure of PA_{63} and the availability of equipment for performing SPR analysis—we decided to reinvestigate the affinities of EF and LF for PA_{63}.

EF gave more consistent data than LF both in SPR measurements and in those on L6 cells. In the SPR measurements, varying the chemistry of tethering of PA_{63} to the BIAcore chip caused no significant differences in the results. Values of k_{on} at 23 °C (averaging 2.8×10^5 M^{-1} s^{-1}) varied within a narrow range, and values of k_{off} (averaging 4.4×10^{-4} s^{-1}) varied by less than a factor of 2. Values of K_d (averaging 1.6 nM) calculated from these rate constants were similarly close. In addition, the values of k_{off} and the calculated K_d from SPR measurements at 4 °C correlated remarkably well with the measured values of these constants obtained with L6 cells at that temperature. This correlation of the results at 4 °C gives us confidence that the SPR results accurately reflect those in a physiological setting and implies that binding of PA_{63} to its receptor does not have a major effect on the affinity for EF.

The constants measured with LF were of the same order of magnitude but varied over a greater range than those with EF. The reason for the variation is unclear, but LF is a Zn^{2+}-dependent protease and shows some tendency to autodegrade.

Our data are consistent with there being a single class of binding sites for EF or LF on the PA_{63} heptamer. The number of EF/LF molecules per heptamer was estimated by SPR to be 1 or 2 (J. L. Elliott, unpublished data). This differs from the previously published stoichiometry of 7 molecules per heptamer (*25*). The reason for this discrepancy is not clear.

The published value for K_d of LF for nicked PA at the cell surface at 4 °C (2×10^{-10} M) (*24*) is virtually the same as our value for the binding of LF to the PA_{63} heptamer (4×10^{-10} M). There are two possible explanations for this. First, the heptameric species might have an equivalent affinity to that of monomeric PA_{63}, assuming the latter remains a discrete entity on the cell surface. Second, and more likely, is the possibility that when nPA preparations are added to cells, PA_{20} dissociates and the oligomer forms rapidly by lateral diffusion of the PA_{63} monomer−receptor complex in the membrane. In the latter case, the values determined by Novak et al. (*24*) were of the interaction of the heptameric prepore.

The similarity in values obtained through amine-coupled and NTA-coupled PA_{63} is notable because the coupling conditions are likely to produce different forms of the heptamer. Amine coupling probably caused the prepore form of the heptamer to convert to the pore form. We assume this because amine coupling required that PA_{63} be injected in a buffer at pH 5.5. For technical reasons, this could not be confirmed. In contrast, NTA coupling requires no acidification step and could be performed at pH 8.0, an environment in which little or no conversion from the SDS-soluble form is observed in solution. The observation that the measured binding constants do not change between these two surfaces suggests that conversion of the prepore form of the heptamer to the pore form has little effect on the affinity of the molecule for EF or LF. This idea is strengthened by experiments that demonstrate the similarity of the off rates of LF_N from receptor-bound PA_{63} oligomers exposed to buffer at pH 8.2 (prepore) and at pH 4.8 (approximately 25% pore). This suggests that there is no appreciable difference between the off rates of LF_N from prepore and pore.

The k_{off} of LF from the pore form of the heptamer was decreased by ~5-fold after the complex had been exposed to acidic buffer and then returned to neutral buffer. The source of this increased affinity is unclear. It may be related to a nonspecific denaturation event. However, it has been reported that EF and LF unfold at low pH, and it is possible that this unfolding enables a tighter association with PA_{63} (*26*).

These findings are puzzling, given that EF and LF are believed to be released into the cytosol during translocation. For EF and LF to be separated from PA_{63}, the strength of the interaction must be diminished, and rapidly, since acid-induced translocation through the plasma membrane has been shown to occur in less than a minute. While the explanation for this discrepancy is not apparent, it should be noted that translocation has not yet been demonstrated in model membrane systems, and it is possible that ancillary proteins (e.g., molecular chaperones) participate in this process in vivo.

The half-life for the dissociation averages ~25 min at 23 °C for the k_{off} constants reported in this work. This is indicative of a stable structure between the heptamer and EF/LF. Such stability enables intoxication in two ways. First, EF and LF are dilute in the extracellular environment, and this tight association between PA_{63} and these ligands results in an effective concentration of toxin at the surface of cells. Also, endocytosis of these complexes is a relatively slow process (*27*), and if the complex were not stable, then EF or LF might be lost to the extracellular medium prior to internalization of the toxin. Thus, the strong affinity of the anthrax toxin components contributes to the overall toxicity of the complex.

The measured values for k_{on} [$(3-5) \times 10^5$ M^{-1} s^{-1}] did not change significantly when the temperature was increased from 4 to 30 °C (Table 2), consistent with the association being diffusion-controlled. The value of k_{off} increased by ~10-fold between 4 and 30 °C, however. The fact that the values at 15 and 23 °C were similar may represent experimental fluctuation. From an Arrhenius plot of the data, we estimated the E_a for the dissociation of EF from PA_{63} to be ~13 kcal/mol. Values of E_a have been measured for dissociation of other tightly bound biological complexes. For example, the dissociation of the human stem-cell-factor dimer to monomer has an E_a of 20.5 kcal/mol (*28*), and the dissociation of high-affinity peptides from the major histocompatibility complex class I K^d molecule has an E_a of 20 kcal/mol (*29*). Thus, the E_a for the dissociation of EF from PA_{63} indicates a tight binding interaction, but the energy barrier for this dissociation is lower than for well-known tight binding interactions.

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