Anthrax Lethal Factor (LF) Mediated Block of the Anthrax Protective Antigen (PA) Ion Channel: Effect of Ionic Strength and Voltage†

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ABSTRACT: The anthrax toxin complex consists of three different molecules, protective antigen (PA), lethal factor (LF), and edema factor (EF). The activated form of PA, PA_{63}, forms heptamers that insert at low pH in biological membranes forming ion channels and that are necessary to translocate EF and LF in the cell cytosol. LF and EF are intracellular active enzymes that inhibit the host immune system promoting bacterial outgrowth. Here, PA_{63} was reconstituted into artificial lipid bilayer membranes and formed ion-permeable channels. The heptameric PA_{63} channel contains a binding site for LF on the cis side of the channel. Full-size LF was found to block the PA_{63} channel in a dose- and ionic-strength-dependent way with half-saturation constants in the nanomolar concentration range. The binding curves suggest a 1:1 relationship between $(PA_{63})_7$ and bound LF that blocks the channel. The presence of a His_6 tag at the N-terminal end of LF strongly increases the affinity of LF toward the PA_{63} channel, indicating that the interaction between LF and the PA_{63} channel occurs at the N terminus of the enzyme. The LF-mediated block of the PA_{63}-induced membrane conductance is highly asymmetric with respect to the sign of the applied transmembrane potential. The result suggested that the PA_{63} heptamers contain a high-affinity binding site for LF inside domain 1 or the channel vestibule and that the binding is ionic-strength-dependent.

The main virulence factors of *Bacillus anthracis* are the poly-D-glutamic acid capsule, which inhibits phagocytosis, and the anthrax toxin complex. The plasmid-encoded tripartite anthrax toxin comprises a receptor-binding moiety termed protective antigen (PA)[1] and two enzymatically active components, edema factor (EF) and lethal factor (LF) (*1*−*3*). EF and LF act on intracellular targets. EF is a calcium- and calmodulin-dependent adenylate cyclase (89 kDa) that causes a dramatic increase of the intracellular cAMP level, altering water homeostasis and intracellular signaling. In addition, EF is believed to be responsible for the edema found in cutaneous anthrax (*2*, *4*, *5*). LF is a highly specific zinc metalloprotease (90 kDa) that removes the N-terminal tail of mitogen-activated protein kinase kinases (MAPKKs). This cleavage initiates still poorly understood mechanisms, leading to the subsequent cell death of some types of macrophages and to the inhibition of the release from macrophages of pro-inflammatory mediators such as nitric oxide, tumor necrosis factor-α, and interleukin-1β (*6*−*8*). More recently, also dendritic cells and T-cells were found to be inhibited by LF and EF (*9*−*11*).

The monomeric anthrax PA is a cysteine-free 83-kDA protein that binds to a ubiquitously expressed integral membrane receptor (ATR) (*11*). PA_{83} bound to ATR is processed by a furin-like protease to a 63-kDa protein PA_{63}. The resulting 20-kDa fragment PA_{20} dissociates from the receptor-bound carboxy-terminal 63-kDa fragment and is released into the extracellular milieu. PA_{63} then spontaneously oligomerizes on the cell surface into a heptamer (*12*) and can bind up to three molecules of EF and/or LF with high affinity ($K_d \sim 1$ nM) (*13*−*15*). The membrane-bound complex of PA_{63} is via clathrin-mediated endocytosis localized inside the endosomes together with bound LF and/or EF (*16*). Heptameric PA_{63} forms under mildly acidic pH of the early endosomes in an ion-permeable channel that plays an important but not yet fully understood role for the translocation of the two toxins in the cytoplasm at the stage of the late endosomes (*7*, *17*).

On the basis of the crystal structure of the water-soluble homoheptameric complex (*12*) and the structure of the structurally related, mushroom-shaped channel of *Staphylococcus aureus* α-hemolysin also formed by a heptamer (*18*), a hypothetical model has been proposed. In this model, the formation of a β-barrel channel requires (a) the unfolding of a Greek-key motif (strands $2\beta_1-2\beta_4$) to form a β-hairpin and (b) the association of seven β-hairpins in the PA_{63} heptamer to form a 14-stranded, membrane-spanning β-barrel (*19*), presumably induced by endosomal acidification.

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[1] Abbreviations: PA, protective antigen; LF, lethal factor; EF, edema factor; *G*, conductance (i.e., current divided by voltage).

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The addition of positively charged compounds such as tetraalkylammonium ions or chloroquine and related compounds is able to block the PA_{63} channel (*20*−*22*). This suggests that the negatively charged PA_{63} channel interacts with positively charged quaternary ammonium ions, which have a structure similar to that of the N-terminal ends of LF and EF. The interaction between a 263-residue N-terminal piece of LF, called LF_N or LF_{263}, and PA_{63} channels has been studied in detail by *in vitro* and also *in vivo* experiments (*23*, *24*). The results suggest that LF_N enters the PA_{63} channels at small positive voltages at the cis side of the membrane and blocks it followed by a translocation process of unfolded LF_N through the channels. A His_6 tag attached to LF_N increases its affinity to the PA_{63} channels (*23*). Deletion of 27 or 36 amino acid residues from the N-terminal end of LF_N inhibits its acid-triggered translocation across the plasma membrane of CHO-K1 cells (*24*). Channel blockage and acid-triggered translocation of truncated LF_N are restored, however, when a His_6 tag is attached directly to the ordered globular portion of the LF_N structure (*24*). This result demonstrated that LF may be translocated with the N-terminal end through the PA_{63} channels in advance.

Here, we show that full-length LF is able to block the PA_{63} channel in a dose- and ionic-strength-dependent way with half-saturation constants in the nanomolar range. The presence of a His_6 tag at the N-terminal end of LF strongly increases the affinity of full-length LF toward the PA_{63} channel. The blockage of the PA_{63}-induced membrane conductance is highly asymmetric with respect to the sign of the applied transmembrane potential, suggesting that the PA_{63} heptamers contain a high-affinity binding site for LF inside the channel vestibule or domain 1.

MATERIALS AND METHODS

Anthrax PA_{63}. Nicked anthrax protein PA_{63} from *B. anthracis* was obtained from List Biological Laboratories, Inc., Campbell, CA. A total of 1 mg of lyophilized protein was dissolved in 1 mL of 5 mM HEPES and 50 mM NaCl at pH 7.5 complemented with 1.25% trehalose. Aliquots were stored at −20 °C.

Cloning, Expression, and Purification of Anthrax LF. The LF wild-type gene was subcloned from pGEX-2Tk LF (*25*) in the *Bam*HI site of the plasmid pRSET A (Invitrogen). The protein was expressed in *Escherichia coli* strain BL21 DE3 (Novagen, Inc.) in a native form, fused to a N-terminal His_6 tag, and purified by FPLC with a Ni-charged Hitrap chelating column (Amersham Biosciences), following the protocol described by the manufacturer. The His_6 tag was removed from His-LF by incubation with enterokinase, leaving five exterior residues, DRWGS, at the N terminus.

Lipid Bilayer Experiments. Experiments with painted lipid bilayers were performed as has been described previously (*26*) using diphytanoyl phosphatidylcholine (Avanti Polar Lipids, Alabaster, AL) as a membrane-forming lipid. The instrumentation consisted of a Teflon chamber with two compartments separated by a thin wall. The membrane hole between the two compartments had a surface area of about 0.4 mm^2. The PA was added from concentrated protein solutions either immediately before membrane formation or after the membranes had turned black. Solvent-depleted bilayers were formed from the same lipid as described (*27*) using a thin Teflon foil with a circular hole of about 50 μm diameter. For successful membrane formation, the hole was pretreated with a solution of hexadecane in hexane. The temperature was maintained at 20 °C during all experiments. The aqueous salt solutions were buffered with 10 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) at pH 6. The PA-induced membrane conductance was measured after application of a fixed membrane potential with a pair of silver/silver chloride electrodes with salt bridges inserted into the aqueous solutions on both sides of the membrane. The electrodes were connected in series to a voltage source and a homemade current−voltage converter made with a Burr Brown operational amplifier. The amplified signal was monitored on a storage oscilloscope (Tektronix 7633) and recorded on a strip chart recorder.

Titration Experiments with LF. The binding of LF and His-LF to the PA_{63} channel was investigated with titration experiments similar to those used previously to study the binding of carbohydrates to the LamB channel of *E. coli* or binding of chloroquine the C2II and PA_{63} channels in single- or multichannel experiments (*22*, *28*, *29*). The PA channels were reconstituted into lipid bilayer membranes. About 30 min after the start of the reconstitution of the channels, the rate of their insertion in the membranes became very small. Then, concentrated solutions of LF or His-LF were added to one or both sides of the membranes while stirring to allow for equilibration. The results of the titration experiments, which resulted in the blockage of the PA_{63} channels, were analyzed in a similar way as performed previously (*28*). The conductance, $G(c)$, of a PA_{63} channel in the presence of LF with the stability constant, K, and the LF concentration, c, is given by the maximum conductance (without LF), G_{max}, times the probability that the LF-binding site in the PA_{63} channel is free

$$G(c) = \frac{G_{max}}{(1 + Kc)} \tag{1}$$

Equation 1 may also be written as

$$\frac{(G_{max} - G(c))}{G_{max}} = \frac{Kc}{(Kc + 1)} \tag{2}$$

which means that the conductance as a function of the LF concentration can be analyzed using Lineweaver−Burke plots. K is the stability constant for LF or His_6-LF binding to the PA_{63} channel. The half-saturation constant, K_S, of its binding is given by the inverse stability constant K^{-1}.

RESULTS

Evaluation of the Stability Constant of the Binding of LF or His_6-LF to the PA_{63} Channel. Previously, we demonstrated that ion-permeable channels formed by the binding components of C2- and Iota-toxins can be blocked by the corresponding enzymatically active components (*30*, *31*). Here, we studied the mechanism of the blockage of the PA_{63}-channel function by anthrax LF in detail. In particular, we were interested in the sidedness of this interaction and in the study of other factors involved in the interaction between LF and PA_{63} channels in *in vitro* experiments. A receptor is required for the binding and oligomerization of PA_{63} on the surface of mammalian cells (*14*). This is not necessary for

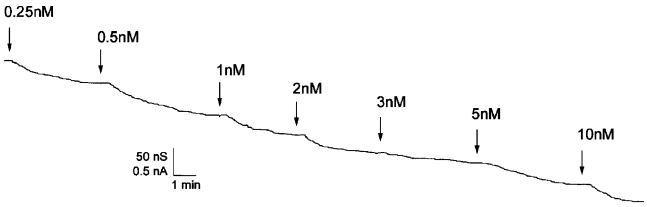

FIGURE 1: Titration of membrane conductance induced by PA_{63} (about 5000 channels) with LF. The membrane was formed from diphytanoyl phosphatidylcholine/*n*-decane. The aqueous phase contained 1 ng/mL PA_{63} protein (added only to the cis side of the membrane), 150 mM KCl, 10 mM MES at pH 6, and LF at the concentrations shown at the top of the figure. The temperature was 20 °C, and the applied voltage was 10 mV. Note that LF blocks only the PA_{63} channels when it is added to the cis side of the membrane.

reconstitution of PA_{63} channels in artificial lipid bilayers, where channel-forming activity is obtained under mildly acidic conditions (*32*). The possible binding of LF to the channel was studied using titration experiments. These measurements allow for the calculation of the stability constants in the case of LF binding to the PA_{63} channels. Figure 1 shows an experiment of this type. Recombinant PA_{63} was added while stirring from the concentrated stock solution (10 μg/mL) to the aqueous phase [concentration $\sim$ 1 ng/mL corresponding to 16 pM for PA_{63} or at a maximum of about 2 pM for $(PA_{63})_7$] on the cis side of a black lipid bilayer membrane. The recombinant protein formed well-defined, cation-selective channels in lipid bilayer membranes as described in different studies (*21*, *22*, *33*). A total of 30 min after the addition of PA_{63}, the rate of conductance increased, caused by the reconstitution of PA_{63} into the membrane, which had slowed considerably. Small amounts of concentrated LF solution were added first to the aqueous phase on the trans side of the membrane (the side opposite to the addition of PA_{63}), with stirring to allow for equilibration (data not shown). The experiments demonstrated that the membrane conductance was not influenced when LF was added to the trans side of the membrane.

On the other hand, the addition of LF to the cis side resulted in a strong decrease of membrane conductance as a function of the LF concentration (see Figure 1), similarly to a recent study of the interaction of a truncated form of LF (LF_{263}) with PA_{63} and its possible translocation through the PA_{63} channel (*23*). The data of Figure 1 and similar experiments with LF were analyzed using Lineweaver–Burke plots according to eq 2. The good fit of the experimental data by the straight line in Figure 2 ($r = 0.982$) suggests indeed that the interaction between LF and the PA_{63} channels represents a single-hit process. From the data of Figure 2, a stability constant, *K*, of $(6.3 \pm 0.4) \times 10^8$ M^{-1} [half-saturation constant $K_S = (1.6 \pm 0.10)$ nM] was calculated from a least-squares fit for the binding of LF to the PA_{63} channel. Similar analyses were performed under a variety of different conditions. The results are included in Table 1. They indicate that LF has a high affinity for the PA_{63} channels because the half-saturation constant for LF binding to the PA_{63} channels was estimated to be about 2.8 nM, in the presence of 150 mM KCl.

We performed some titration experiments with solvent-depleted membranes of the Montal–Muller type (*27*) to see if the solvent has any influence on the binding of LF to the

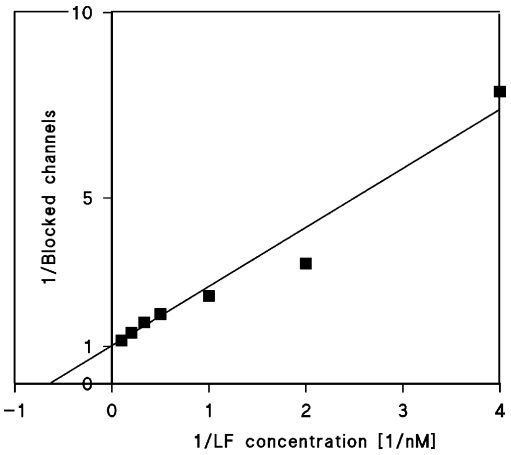

FIGURE 2: Lineweaver–Burke plot of the inhibition of the PA_{63}-induced membrane conductance by LF. The straight line was obtained from a linear regression of the data points ($r = 0.982$) and corresponds to a stability constant, *K*, for LF binding to PA_{63} of $(6.3 \pm 0.4) \times 10^8$ M^{-1} [half-saturation constant, $K_S = (1.6 \pm 0.10)$ nM].

Table 1: Stability Constants, *K*, for the Inhibition of Channel Formation by PA by LF and His_6-LF in Lipid Bilayer Membranes[a,b]

LF			His_6-LF		
ionic strength (mM)	*K* (10^8 M^{-1})	K_S (nM)	ionic strength (mM)	*K* (10^8 M^{-1})	K_S (nM)
50	23	0.44	50	32.0	0.32
150	3.60	2.80	150	55.0	0.18
300	0.45	22	300	42.0	0.24
1000	0.046	220	1000	5.4	1.90

[a] The data represent means of at least three individual titration experiments. The standard deviation was typically less than 20% of the mean values. K_S is the half-saturation constant. [b] The membranes were formed from diphytanoyl phosphatidylcholine/*n*-decane. The aqueous phase contained the indicated KCl concentration and about 1 ng/mL PA_{63}; $T = 20$ °C.

PA_{63} channel. The results demonstrate that *n*-decane had no influence on LF binding. The mean value for LF binding to PA_{63} channels at 150 mM KCl and 10 mM MES at pH 6 was 2.1 nM [$\pm$0.6 nM standard deviation (SD), with a mean of four experiments]. This result indicated that the type of membranes used for the titration experiments, either solvent-depleted or solvent-containing, had a very small influence, if any, on LF binding to the PA_{63} channels.

LF Binding to PA_{63} Is Ionic-Strength-Dependent. Previous studies suggested that a variety of positively charged molecules including tetraalkylammonium ions and 4-aminiquinolones are able to block the conductance of PA_{63} channels (*20*–*22*). This could mean that at least part of the binding between LF and the PA_{63} channel is caused by an interaction between oppositely charged groups localized at both molecules. Such an interaction should be ionic-strength-dependent. To test this possibility, we also performed titration experiments at different ionic strength. The results of these measurements suggested indeed that the stability constant of LF binding to the PA_{63} channels was ionic-strength-dependent (see Table 1). The half-saturation constant for LF binding increased from 50 mM to 1 M KCl by about a factor of 500.

His_6-LF Has a Higher Affinity for the PA_{63} Channel. LF was expressed in *E. coli* with a His_6 tag at the N-terminal end to simplify purification. For most of the experiments

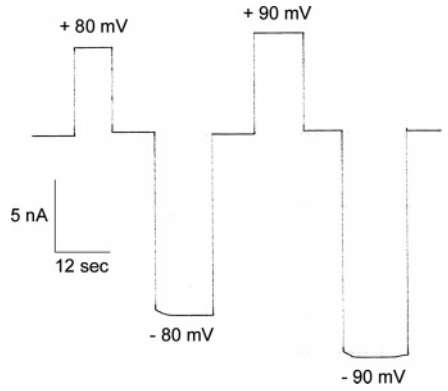

FIGURE 3: Asymmetric current response upon application of short voltage pulses of ±80 and ±90 mV to a diphytanoyl phosphatidylcholine/*n*-decane membrane in the presence of 2 nM LF added to the cis side of the membrane. The aqueous phase contained 1 ng/mL PA_{63} protein (added only to the cis side of the membrane), 150 mM KCl, and 10 mM MES at pH 6. The temperature was 20 °C. Note that the current through the PA_{63} channels was highly asymmetric with respect to the sign of the applied voltage.

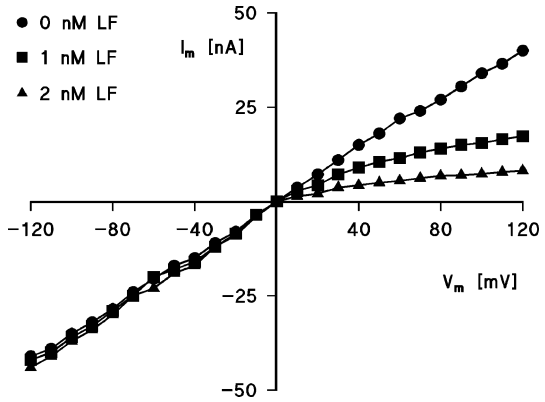

FIGURE 4: Current voltage relationships of open PA_{63} channels (full points) and PA_{63} channels blocked with different concentrations of LF, 0 nM (●), 1 nM (■), and 2 nM (▲). The aqueous phase contained 1 ng/mL PA_{63} protein (added only to the cis side of the membrane), 150 mM KCl, and 10 mM MES at pH 6. The temperature was 20 °C. The sign of the voltage corresponds to that on the cis side of the membrane.

reported here, the His_6 tag was removed by protease treatment using enterokinase. His_6-LF exhibited an even higher affinity for binding to the PA_{63} channel. The half-saturation constant for His_6-LF binding to the PA_{63} channel was on average 0.18 nM ($K = 5.5 \times 10^9$ M^{-1}) at an ionic strength of 150 mM KCl, which means that the stability constant for binding for His_6-LF was approximately a factor of 10 higher than that without the His_6 tag (see Table 1), which was already found in experiments with an N-terminal form of LF, LF_N (LF_{263}) (*23*, *24*). Similarly, as with LF, binding of His_6-LF to the PA_{63} channels was also ionic-strength-dependent, although it was less pronounced as in the case of LF. These results support the view that the interaction of LF with the PA heptamer is based on the ion−ion interaction between the PA_{63} channels and the N-terminal end of LF (*23*, *24*) to which, at pH 6, the positively charged His_6 tag obviously contributes.

Binding of LF to the PA_{63} Channel Is Highly Asymmetric with Respect to the Sign of the Applied Membrane Potential. The titration experiments described above were performed with small positive potentials of 10−20 mV applied to the cis side of the membrane. For small negative voltages at the cis side, no binding of LF could be detected. This result indicated that the binding of LF to the PA_{63} channel was highly asymmetric with respect to the sign of the applied membrane potential, whereas that through the open PA_{63} channels was almost symmetrical with the exception of slow channel inactivation at low ionic strength and high negative potentials (*21*, *22*). The asymmetric response of the interaction between LF and the PA_{63} channels was studied in detail. Figure 3 shows an experiment in which short voltage pulses of ±80 and ±90 mV were applied to the cis side of a membrane containing about 1000 PA_{63} channels and 2 nM LF was added to the cis side of the membrane. The current response to the applied voltage was highly asymmetric with respect to the sign of the applied voltage. When the voltage was applied for a longer time to the trans side of the membrane, the current showed decreasing behavior probably because of slow PA_{63} channel closure (*22*−*24*). The application of high positive potentials to the cis side resulted, in some experiments, in a current increase (see below). Figure 4 shows current−voltage curves of the open and LF-induced

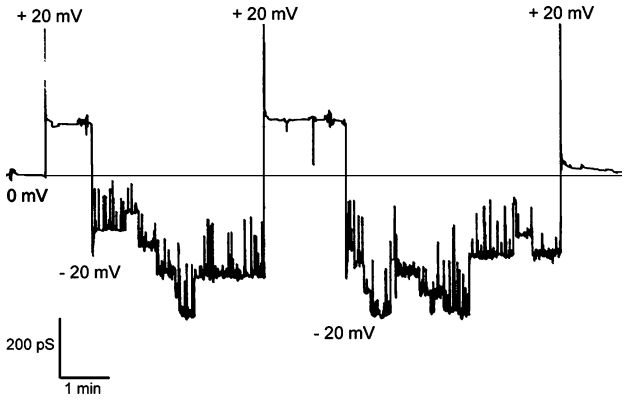

FIGURE 5: Asymmetric current response upon application of voltages of ±20 mV to a diphytanoyl phosphatidylcholine/*n*-decane membrane, which contained 4−5 PA_{63} channels. LF (1 nM) was added to the cis side of the membrane. The aqueous phase contained 0.01 ng/mL PA_{63} protein (added only to the cis side of the membrane), 150 mM KCl, and 10 mM MES at pH 6. The temperature was 20 °C. Note that the current through the PA_{63} channels decreased only when the cis side, the side of the addition of PA_{63} and LF, was positive. The open PA_{63} channels showed some flickering for negative voltage applied to the cis side.

partially closed PA_{63} channels at two different LF concentrations. The results indicated that the current at positive potential decreased when LF was present at the cis side of the membrane. A higher concentration of LF resulted in a higher blockage of the channels when positive potentials were applied to the cis side. No channel block was observed for negative potentials at the cis side for short voltage pulses (see Figure 4). PA_{63} channel blockage could also be observed in single-channel experiments similar to that shown in Figure 5. Five PA_{63} channels were reconstituted into a lipid bilayer membrane, and 1 nM LF was added to the cis side of the membrane. A total of 20 mV applied to the cis side resulted in almost complete closure of all channels, whereas the channels are most of the time open when negative potential was applied to the cis side.

Binding of LF to the PA_{63} Channels Is Voltage-Dependent. The current−voltage relationships of Figure 4 and of similar experiments suggested that binding of LF to the PA_{63} channel occurs in a voltage-dependent way. To study this process in more detail, PA_{63} was reconstituted in a lipid bilayer

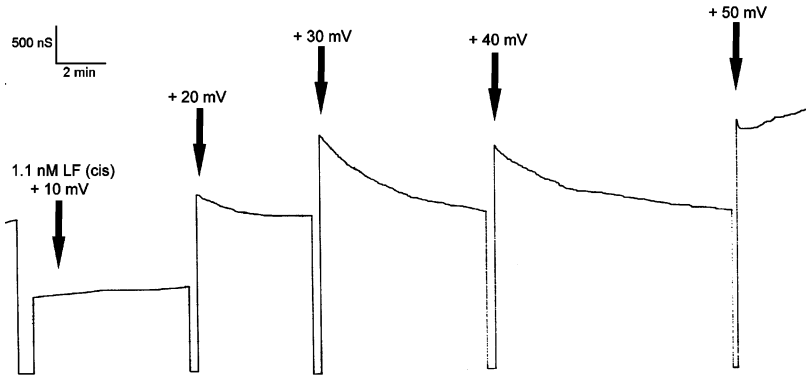

FIGURE 6: Voltage dependence of PA_{63} channel blockage with 1.1 nM LF added to the cis side of the membrane. The PA_{63}-induced membrane conductance was titrated with 1.1 nM LF that caused a decrease of the initial current by about 60%. Then, voltage steps with an increasing voltage and a positive sign were applied to the cis side. The aqueous phase contained 1 ng/mL PA_{63} protein (added only to the cis side of the membrane), 150 mM KCl, and 10 mM MES at pH 6. The temperature was 20 °C. Note that the voltage steps at 20, 30, and 40 mV were all followed by an exponential decrease of the current, indicating that the stability constant *K* for LF binding to the PA_{63} channels increased.

membrane. When the membrane conductance became stationary, LF was added to the cis side of the membrane at a concentration of 1.1 nM while stirring to allow for equilibration. Then, the experiment of Figure 6 started. Positive voltage pulses were applied to the cis side of a membrane after the blockage of a considerable part of the channels. Whereas 10 mV was not able to induce a further blockage of the channels, higher voltage pulses starting with 20 mV resulted in a considerable further decrease of the PA_{63}-induced membrane conductance. This result indicated that channels, which were not blocked before by LF, started to close as a result of the higher voltage, pointing to an increase of the stability constant of binding. Starting with about 50−60 mV, the current started to increase again (see Figure 6). This effect has previously been explained by the translocation of a truncated form of LF through the PA_{63} channels (*23*) because the truncated LF has been removed from the cis side before the application of the voltage pulses. However, because we did not remove LF from the cis side, the translocation of LF (generating free binding sites for LF at the PA_{63} channels) cannot account for the increase of the conductance. It seems moreover that this effect has to do with voltage-induced reconstitution of PA_{63} channels because it was only observed when membrane voltage was increased in steps from low to high voltage. When at first high voltage was applied to the membranes, the conductance increase was small or absent (data not shown).

DISCUSSION

Stability Constants for LF Binding to the PA_{63} Channel Are Extremely High. In previous studies, we already demonstrated that the enzymatic components of C2- and Iota-toxins bind to the binding components (C2II) of C2-toxin from *Clostridium botulinum* and Iota b of *Clostridium perfringens* and inhibit channel formation *in vitro* (*29*, *30*). The PA of anthrax toxin shares significant sequence identity (33%) with C2II, suggesting that the two proteins have similar modes of action. PA_{63} has been crystallized in its monomeric and heptameric prepore form (*12*). The heptameric form is the one that binds to the target cell membrane and inserts a 14-stranded β-barrel into the membrane. The mushroom-shaped channel-forming complex of PA_{63} is highly asymmetric, because most hydrophilic material is localized on one side of the membrane; the cis side of lipid bilayer membranes, the surface of the target cell, or the inside of the endosomes and only a very small part of the oligomer are localized in the target cell membrane (*34*). Its structure is presumably similar to that of α-toxin of *Staphylococcus aureus*, which also forms a heptamer with some sort of vestibule on the cis side of the membrane (*18*).

Here, we studied the interaction between full-length LF and the PA_{63} channel in detail. Titration experiments were performed with membranes that contained a large number of PA_{63} channels, and LF was added to the aqueous phase. The results suggest an extremely high affinity of LF to the PA_{63} channels. The half-saturation constant for LF binding at an ionic strength of 150 mM KCl, which should be close to the *in vivo* situation, was on average about 2.8 nM. The half-saturation constant was not dependent upon the type of membranes, which means that PA_{63} channels reconstituted in solvent-depleted and solvent-containing membranes showed the same affinity to LF. The half-saturation constant of 2.8 nM, measured in this study, at an ionic strength of 150 mM KCl has to be compared with an equilibrium constant, K_d, calculated from kinetic data of LF binding to amine-coupled PA_{63}, in surface plasmon resonance (SPR) measurements, which yielded 2.8 nM for LF at 150 mM NaCl, whereas for NTA-coupled PA_{63}, an equilibrium constant, K_d, of 11 nM was observed (*15*). This means that the results of the titration experiments at low voltage described here are in excellent agreement with data from SPS measurements with LF and LF_N derived earlier (*15*). EC_{50} toxicity of the LF protein on PA-treated J774A.1 macrophages was determined to be at a much lower concentration (6.1 ng/mL corresponding to about 0.07 nM) (*35*). However, LF binding to PA was also measured in the same study on J774A.1 cells using the competition with radiolabeled native LF. Half-saturation for LF binding was obtained at a competitor concentration of about 150 ng/mL (*35*). This means that half-saturation for LF binding to PA is obtained at about 1.7 nM under these conditions, again in good agreement with our data and results of the SPR measurements (*15*). The similarity in the binding constants determined by titration experiments with lipid bilayers, SPR, and measurements on the surface of different cells suggests that the cell-surface receptor for PA (*11*) does not modulate LF binding to PA_{63}. Furthermore, it is clear

from a comparison of the results derived from experiments with full-length LF with those derived from the binding of LF_N to the PA_{63} channel that binding of LF to PA is localized within the first 263 amino acid residues of PA (*23*, *24*), which means that there is not much difference between full-length LF and LF_N.

Our data suggest that the interaction between full-length LF and PA_{63} is ionic-strength- and voltage-dependent (see below), which has not been studied thus far. The results suggest that charges of opposite sign on both molecules are involved in the binding of LF to the PA_{63} channel. Both the effect of voltage and ionic strength on LF binding to the PA_{63} channel may explain why the half-saturation constant for LF binding to PA_{63} was lower than ours in a recent study because the ionic strength was 100 mM and the applied voltage was 50 mV (*36*). Although it has been reported in the literature that up to three molecules of LF and/or EF can bind to the PA_{63} channel (*13*, *37*−*39*), it is clear from the data presented here that LF-mediated PA_{63} channel blockage is a single-hit mechanism, otherwise we should have observed deviations from the Langmuir adsorption isotherm. This means that the first LF molecule bound to the binding site leads already to channel blockage irrespective of the maximum number of binding places for LF on the PA_{63} channel, which is three (*38*). It is interesting to note that the transport efficiency of LF into the target cells is independent from the number of bound LF to the PA heptamer, indicating the independent translocation of LF (*39*).

The high affinity of LF for the PA_{63} channel may interfere with the half-saturation constant because some of the LF may be lost because it is bound to $(PA_{63})_7$ in the aqueous phase. However, the concentration of PA_{63} in the aqueous phase of the cis side was about 16 pM, which means that the concentration of heptamers could be at maximum around 2 pM. This concentration is not high enough to interfere with the results of titration experiments with LF, where half-saturation constants dependent upon ionic strength between 440 pM and 220 nM were found. Some LF may be lost to the walls of the cell membrane. However, we found in control experiments no indication for such a loss.

His_6 Tag Increases LF Binding to the PA_{63} Channel. LF was produced by the heterologous expression in *E. coli* and contained for easy purification an N-terminal His_6 tag. Some experiments reported here were performed with this construct. Full-length His_6-LF showed a much higher affinity for the PA_{63} channel than LF alone, which was also observed for a truncated form of LF (LF_N or LF_{263}) (*23*, *24*). This result suggested that the His_6 tag is somehow involved in the binding of His-LF to the PA_{63} channel and increases binding affinity, which may be caused by the positively charged groups of the His_6 tag. At pH 6 close to the pK_a of histidine, at least one of the six imidazol groups should be charged, even taking into account that the charge of a protonated imidazol residue may influence the pK_a of the neighbors. This result agrees with previous reports that His_6-LF and also LF bind with their N termini to the PA_{63} heptamers, otherwise the increased affinity for His-LF binding cannot be understood (*23*, *24*). Similarly, His_6 tag fused to the N terminus of N-terminal deletion constructs of LF_N (lacking 27, 36, or 39 amino acids) restored translocation efficiency to wild-type levels (*24*). Similar conclusions based on a different experimental approach are discussed in a recent study (*22*). Evidence that quaternary ammonium groups may be involved in the binding of LF and EF to the PA_{63} channel comes also from other studies. In particular, it has been demonstrated that tetraalkylammonium ions block the PA_{63} channel (*20*, *21*). Similarly, 4-aminoquinolones such as chloroquine, quinacrine, and fluphenazine have an even higher affinity to the PA_{63} channel with half-saturation constants in the micro−nanomolar range dependent upon the ionic strength of the aqueous solutions (*22*). All of these compounds are more effective from the cis side, which suggests that they bind to negatively charged groups in the vestibule or domain 1. In addition, recent investigations performed with cysteine mutants of PA revealed that, upon binding of MTS-ET reagents to mutated PA, binding of the positively charged channel blocker tetrabutylammonium (TBA) from the trans side of the protein is blocked. Nassi and co-workers (*19*) demonstrated that, after S349C mutant channels had reacted with MTS-ET, subsequent addition of TBA to the cis side produced the usual 3.5-fold decrease in conductance, whereas the MTS-ET reaction prevented *trans*-TBA from producing its usual 2-fold decrease in conductance. This result suggested that the TBA-binding site is located somewhere in the cap region of the PA_{63} channel. It is noteworthy that chloroquine that binds also to this vestibule blocks probably also the PA_{63} channel *in vivo*, because it enhances survival in *B. anthracis* intoxication (*40*).

Voltage-Dependent Blockage of PA_{63} Channels by LF. The binding of LF was highly asymmetric with respect to the sign of the applied membrane potential as was also shown in a recent study suggesting a diode-like behavior of the PA channel (*36*). Channel blockage was only observed when the side of the addition of PA_{63} and LF, the cis side, was positive. Negative potential at the cis side had no effect on the LF-mediated block of the PA_{63} channels. Only some minor channel inactivation was observed at low ionic strength and high negative voltages irrespective of the LF addition, similar to that described previously (*20*, *22*, *33*). Furthermore, increasing positive voltages applied to the cis side of the membrane led to somewhat increasing stability constants for binding, reaching a voltage-independent plateau value at higher voltages (*36*). This may hint to a voltage-dependent translocation of a truncated form of LF (LF_{263}) through the PA_{63} channel because the truncated form can be washed out from the cis side, indicating that a PA_{63} channel can be open again after translocation of LF_{263} (*23*). However, such an explanation cannot account for the conductance increase observed here because there was enough LF around to block the PA_{63} channel once when the binding site was freed by the transport of LF through the channel. On the other hand, it is clear that LF senses a considerable fraction of the voltage, at variance from our previous study with 4-aminoquinolones, where the voltage drop across their binding site was small (*22*). Here, the effect of the voltage on LF binding was much higher, which indeed suggested that a certain part of LF is localized inside the channel and not only in the vestibule.

Charge Effects of LF Binding to the PA_{63} Channel. The data shown in Table 1 demonstrate a considerable dependence of LF binding on the bulk aqueous KCl concentration. This result indicates that charge effects influence the interaction of the PA_{63} channel with LF and His-LF. These charge

effects are caused by negatively charged groups probably localized in the vestibule or domain 1 of the channel and their interaction with positive charges at the N-terminal end of LF (*13*, *36*, *41*). Charges within or near the PA_{63} channel result in a substantial ionic-strength-dependent potential, which attracts cations and repels anions. A quantitative description of the effect of charges on the binding of LF to the PA_{63} channel may be attempted according to the Debye−Hückel theory describing the effect of charges in an aqueous environment. In the case of a negative charge, q, in an aqueous environment, a potential ϕ is created that is dependent upon the distance, r, from the charge

$$\Phi = \frac{qe^{-(r/l_D)}}{4\pi\epsilon_0\epsilon r} \quad (3)$$

ϵ_0 (=8.85 × 10^{-12} F/m) and ϵ (=80) are the absolute dielectric constants of vacuum and the relative constant of water, respectively, and l_D is the so-called Debye length that controls the decay of the potential (and of the accumulated positively charged ions) in the aqueous phase

$$l_D^2 = \frac{\epsilon\epsilon_0 RT}{2F^2 c} \quad (4)$$

where c is the bulk aqueous salt concentration, R is the gas constant [R = 8.31 J/(mol °C)], T is the absolute temperature (T = 293 K), and F is Faraday's constant (F = 96 500 As/mol). The negative potential created by the negative charges in or near the channel has important implications on the concentration of countercharges (*22*, *29*), similar to the effect of charges inside ion channels (*42*−*44*). The concentration of the monovalent cations near the charges increases because of the negative potential. Their concentration, c_0^+, at the channel is dependent upon the potential Φ and given by

$$c_0^+ = ce^{-(\phi F)/RT} \quad (5)$$

In the following, we assume that the negative charges are localized on the cis side of the PA_{63} channel, which is the binding site for LF. This means that the accumulated positively charged N terminus of LF virtually increases the stability constant for LF binding as a function of the ionic strength j in the aqueous phase

$$K(c) = K^* c_0^+/c \quad (6)$$

where K^* is the stability constant for the binding reaction when no negative charges influence the binding process. Equation 5 can be introduced into eq 6, and we can try to fit the on rate of LF binding to the PA_{63} channel given in Table 1. A best fit of the data was obtained using eqs 3−6 by assuming that six negatively charged groups (q = −9.6 × 10^{-19} As) are located within the channel vestibule and that its radius is approximately 0.7 nm. The results of this fit are shown in Figure 7 for eq 6. The solid line represents the fit of the stability constant of binding versus ionic strength (i.e., the KCl concentration) by using the Debye−Hückel theory and the parameters mentioned above together with a stability constant for binding K^* = 1.6 × 10^7 mol^{-1} when no charge is around. Figure 7 demonstrates that the influence of the surface charges is rather small at high ionic strength, c, i.e., small l_D (see eq 4). It has to be noted that the number of negative charges involved in the accumulation of cations within the channel have to be considered as tentative because the relative dielectric constant in the vicinity of the charge is not known. This has been discussed in detail elsewhere (*45*, *46*). On the other hand, the diameter of the channel opening in the vicinity of the charges appears to be more precise.

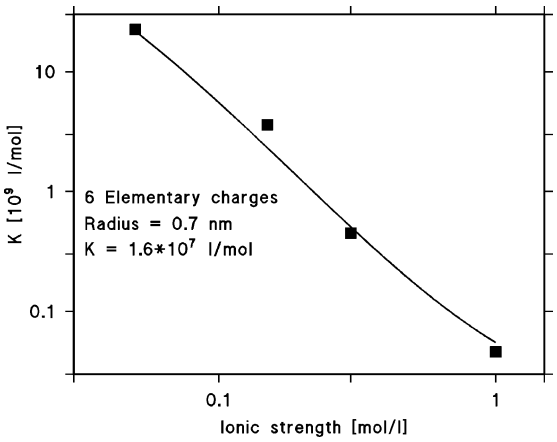

FIGURE 7: Stability constant of LF binding to the PA_{63} channel as a function the ionic strength (equal to the KCl concentration) in the aqueous phase. The solid line shows the fit of the stability constants as a function of the ionic strength of the aqueous phase using eqs 3−6 and assuming that six negatively charged groups (q = −9.6 × 10^{-19} As) are located within the channel and that its radius is approximately 0.7 nm.

The considerations made above suggest that ion−ion interactions contribute substantially to the binding of LF to PA. These interactions presumably have to do with some of the amino acids in LF, which are known by alanine-scanning mutagenesis to be responsible for LF binding to PA_{63} (*47*). These amino acids are D182, D187, L188, Y223, H229, L235, and Y236, whose mutations result in significant binding defects of LF to PA_{63}. Additional amino acids have been identified in a recent study where the complex between LF_N and PA_{63} was studied in detail (*48*). In particular, pairs of charged residues were identified in the two proteins that could be reversed without an effect on the complex. The pairs of charge-reversal mutants are LF_N D187K−PA K213D/K213E and LF_N E142K−PA K218E (*48*), which means that they are involved in electrostatic interactions and could account for the ionic-strength effect studied here. The amino acids on PA_{63} responsible for LF binding are also known in detail. However, K397 and F427 may be involved in pore formation of PA_{63} and subsequent LF translocation in cells (*49*, *50*) but are presumably not involved in the ionic-strength effects described here. Other possible candidates for the interaction between PA are E398 and D425 (see the table of contents graphic), which means that a further mutation of PA and LF has to be undertaken to further increase our knowledge on the points of the interaction between LF and PA.

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