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# Anthrax toxin complexes: heptameric protective antigen can bind lethal factor and edema factor simultaneously

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**Abstract**

The 83 kDa protective antigen ($PA_{83}$) component of anthrax toxin, after proteolytic activation, self-associates to form ring-shaped heptamers ($[PA_{63}]_7$) that bind and aid delivery of the Edema Factor (EF) and Lethal Factor (LF) components to the cytosol. Here we show using fluorescence (Förster) resonance energy transfer that a molecule of $[PA_{63}]_7$ can bind EF and LF simultaneously. We labeled EF and LF with an appropriate donor/acceptor pair and found quenching of the donor and an increase in sensitized emission of the acceptor when, and only when, a mixture of the labeled proteins was combined with $[PA_{63}]_7$. Addition of unlabeled $PA_{63}$-binding domain of LF to the mixture competitively displaced labeled EF and LF, causing a loss of energy transfer. In view of the known maximum occupancy of 3 ligand molecules per $[PA_{63}]_7$, these findings indicate that PA, EF, and LF can form mixtures of liganded toxin complexes containing both EF and LF.


*Keywords:* Anthrax toxin; Edema factor; Lethal factor; Protective antigen; Stoichiometry; Ligand binding; Macromolecular interactions

Anthrax toxin consists of three non-toxic proteins that interact at the mammalian cell surface to form toxic non-covalent complexes [1,2]. The three proteins are: edema factor (EF), a calmodulin dependent adenylate cyclase; lethal factor (LF), a $Zn^{2+}$-metalloprotease; and protective antigen ($PA_{83}$, 83 kDa), a protein that binds the enzymatic components and delivers them to the cytosol of mammalian cells. Once within the cytosol, EF and LF contact their substrates and catalyze reactions that result in toxicity [3–5].

The current, generally accepted, model of anthrax toxin assembly and action is as follows. $PA_{83}$ binds to either of two known cell surface receptors, anthrax toxin receptor/tumor endothelial marker-8 (ATR/TEM8) and capillary morphogenesis protein 2 (CMG2) [6,7]. A cellular protease of the furin family then cleaves $PA_{83}$ into two fragments: $PA_{20}$ (20 kDa), corresponding to the N-terminus, and $PA_{63}$ (63 kDa), corresponding to the C-terminus [8,9]. $PA_{20}$ dissociates into the medium and plays no known further role in toxin action. Removal of $PA_{20}$ eliminates a steric barrier to self-association, allowing $PA_{63}$ to oligomerize and form a heptameric ring-shaped structure ($[PA_{63}]_7$), termed the prepore [10]. This structure can bind up to three molecules of LF or EF competitively and with nanomolar affinity [11,12]. The resulting complexes are localized to detergent-resistant lipid microdomains, where they undergo receptor-mediated endocytosis [13]. Acidification of the toxin-containing compartment then causes a structural rearrangement of the prepore that enables it to insert into the membrane, forming a pore [14,15]. Pore formation is linked to translocation of the bound enzymatic cargo (LF or EF) to the cytosol. LF has been shown to cleave members of the mitogen-activated protein kinase kinase family [4,5], but the events leading to death are poorly defined. Elevation of cAMP concentration by

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EF may protect the bacteria from degradation by macrophages [16].

EF and LF have N-terminal $PA_{63}$-binding domains and similar binding affinities for heptameric $PA_{63}$ (in the low nanomolar range) [11]. Mogridge et al. [12] used multi-angle light scattering coupled with size exclusion chromatography to demonstrate that $[PA_{63}]_7$ binds a maximum of three molecules EF or $LF_N$, the $PA_{63}$-binding domain of LF, at saturation. The footprint of EF/LF is such that only alternating sites, and hence three of the seven sites in the symmetric $[PA_{63}]_7$, could be occupied simultaneously. Although these findings suggest that EF and LF can bind simultaneously to the same heptameric $PA_{63}$ molecule, it has not been ruled out that co-binding could be prevented due to steric clash or another mechanism. Here we demonstrate using FRET as a metric that EF and LF can in fact co-bind to $[PA_{63}]_7$.

## Materials and methods

*Production and purification of proteins.* The production and purification of $PA_{83}$, LF, and EF have been described previously [17]. Briefly, the proteins were expressed using pET-22b(+) ($PA_{83}$) and pET-15b (LF and EF) in *Escherichia coli* in 5- or 10-L fermentors using ECPM1 media [17,18]. $PA_{83}$ was purified from periplasmic lysates by ion exchange and size exclusion chromatography. Hexahistidine tagged LF and EF were purified from bacterial lysates by metal chelating affinity and size exclusion chromatography. Following chromatographic purification, proteins were concentrated in centrifugal concentrators. All purified proteins were judged to be more than 90% pure by SDS–PAGE with Coomassie blue staining. Protein concentrations were determined by absorbance spectrophotometry, assuming extinction coefficients of 75,670 $cm^{-1}M^{-1}$ ($PA_{83}$), 77,660 $cm^{-1}M^{-1}$ (EF), and 73,250 $cm^{-1}M^{-1}$ (LF). $[PA_{63}]_7$ was produced and purified by first nicking $PA_{83}$ with TPCK-treated trypsin at a 1:1000 w/w ratio for 35 min at room temperature followed by addition of soybean trypsin inhibitor at a 1:100 w/w ratio. This nicked $PA_{83}$ oligomerized on an ion exchange column and eluted as $[PA_{63}]_7$ at greater than 95% purity as judged by PAGE and Coomassie blue staining. Proteins were aliquoted and stored at −80 °C.

*Protein labeling with fluorescent dyes.* EF and LF were labeled with either the amine-reactive dye Alexa 488 tetrafluorophenyl ester or Alexa fluor 594 succinimidyl ester (Molecular Probes). The proteins were first exchanged into 200 mM carbonate buffer, pH 8.5, using size exclusion chromatography and then reacted with a molar excess of dye for 1 h at room temperature. Similarly, LF and EF containing single-cysteine mutations were labeled with the cysteine-reactive probes Alexa fluor 488 $C_5$ maleimide or Alexa fluor 594 $C_5$ maleimide (Molecular Probes). Any disulfide bonds were reduced by incubating in 20 mM DTT on ice for 30 min. Gel filtration was used to remove DTT and to change the buffer to 50 mM Tris–HCl, 150 mM NaCl, pH 7.2, for the labeling reaction. Molar excesses of the dye were added to the protein solutions and allowed to react for 2 h at room temperature. In all cases, free dye was largely removed by concentration using centrifugal protein concentrators, and the protein was exchanged back into 50 mM Tris–HCl, pH 8.5, buffer by gel filtration. Protein concentration and dye to protein ratio were determined using absorbance spectrophotometry. Labeled protein was stored at −80 °C.

*FRET measurements between LF and EF bound to $PA_{63}$ oligomer.* Mixtures of labeled LF and EF (LF*594 SE and EF*488 TFP or LF E126C*546 and EF G241C*488; 330 nM total concentration of EF + LF) were added to cuvettes containing 50 mM Tris–HCl, pH 8.5, 150 mM NaCl, and 0.5 mg/ml BSA at 20 °C in the presence or absence of 100 nM $[PA_{63}]_7$ and allowed to incubate for >15 min. Fluorescence emission spectra were recorded from 500 to 650 nm with 1 nm steps, 0.3 s integration time, and a 4 nm bandpass. The 488 nm line from an air-cooled argon-ion laser was used for excitation. Integrated fluorescence emission intensities were measured at 615 ± 10 and 525 ± 10 nm, and the values were divided to yield an apparent FRET efficiency.

The competition assay for EF and LF co-binding to $[PA_{63}]_7$ was carried out under the conditions and concentrations described above. Kinetic transients were recorded by simultaneously measuring the ratio of emission intensities at 615 ± 8 and 525 ± 8 nm following addition of 10 μM of the N-terminal binding domain of LF. The kinetic transient was fitted to exponential decay models using non-linear least squares algorithms to verify exponential kinetics.

## Results and discussion

We investigated whether EF and LF simultaneously bind to a single $[PA_{63}]_7$ molecule using FRET between donor-labeled EF and acceptor-labeled LF. Under our experimental conditions, no resonance energy transfer should be observed between EF and LF in the absence of $[PA_{63}]_7$. Similarly, labeled EF and LF bound to different $[PA_{63}]_7$ molecules should not show resonance energy transfer, due to the distance dependence of FRET, where acceptor sensitized emission is only measurable to approximately two times the Förster distance (or approximately 110–120 Å; Förster distance for the fluorophores used in these experiments is 55–60 Å) [19]. Therefore, energy transfer reflects the presence of EF and LF bound to the same $[PA_{63}]_7$ molecule.

Samples, prepared in triplicate, contain 100 nM $[PA_{63}]_7$, 230 nM LF*594 TFP, and 100 nM EF*488 SE. A donor or acceptor alone control, also in triplicate, contained 100 nM $[PA_{63}]_7$ and either 100 nM EF*488 TFP or 230 nM LF*594 SE. Samples were incubated at least 15 min at room temperature prior to measurement, a period sufficient for almost complete equilibration, based on previously determined on-rates for EF- and LF-$[PA_{63}]_7$ interactions and the concentrations used [11]. Kinetic transients monitoring FRET between EF*488 TFP and LF*594 SE on $[PA_{63}]_7$ association kinetics (data not shown) confirmed that equilibrium was approached within 15 min. Emission spectra from 500 to 650 nm were recorded for each sample using 488 nm excitation. Fig. 1A shows emission spectra of donor alone (dotted; EF alone) and donor plus acceptor (solid; EF + LF) in the presence of $[PA_{63}]_7$ where the direct excitation of the acceptor has been subtracted. A low but significant level of sensitized emission is observed (approximately 5–7% FRET efficiency) indicating the presence of both EF and LF bound to the same $[PA_{63}]_7$ molecule. Fig. 1B shows corresponding spectra in the absence of $[PA_{63}]_7$, where the emission spectrum of the donor alone (dotted; EF) is compared to those of donor plus acceptor (solid; EF + LF) and the direct excitation has been subtracted. These spectra show no

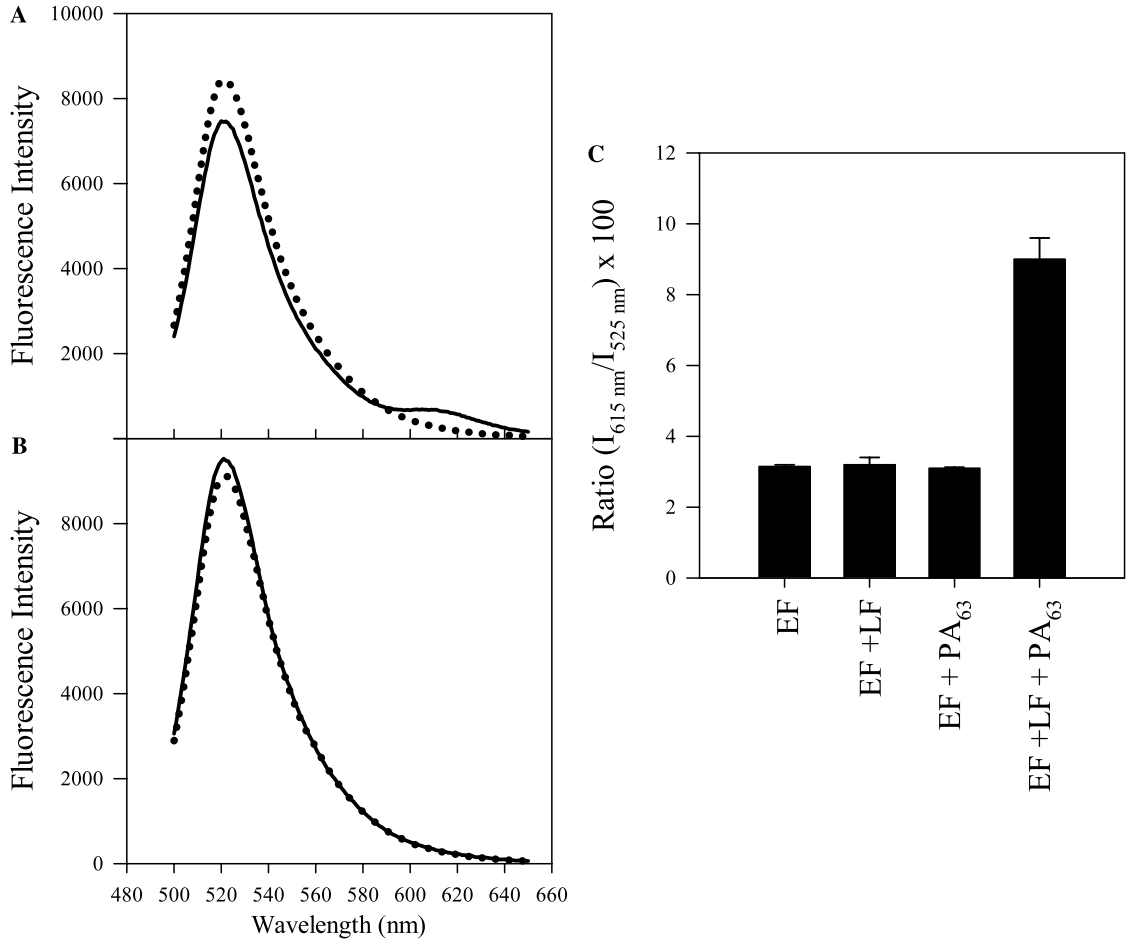


Fig. 1. Sensitized emission spectra of EF and LF bound to $[PA_{63}]_7$. (A) Fluorescence emission spectra of 100 nM $[PA_{63}]_7$ and 100 nM EF*488 TFP (dotted; donor alone) and 100 nM $[PA_{63}]_7$, 100 nM EF*488 TFP, and 230 nM LF*594 SE (solid; donor plus acceptor). An acceptor alone spectrum (100 nM $[PA_{63}]_7$ and 230 nM LF*594 SE) was first subtracted from the donor plus acceptor spectrum (solid). (B) Fluorescence emission spectra of 100 nM EF*488 TFP (dotted; donor alone) and 100 nM EF*488 TFP plus 230 nM LF*594 SE (solid; donor plus acceptor). An acceptor alone spectrum (230 nM LF*594 SE) was first subtracted from the donor plus acceptor spectrum (solid). (C) Ratiometric analysis of spectral data in (A) and (B). Ratios of the integrated intensity of the fluorescence emission at 615 ± 10 and 525 ± 10 nm were calculated for each condition. The ratio values shown have had the direct excitation of the acceptor alone subtracted prior to calculating the ratio. Error bars show the standard deviation.

evidence of sensitized emission. The two sets of spectral data are quantified in Fig. 1C as a bar graph that demonstrates the level and significance of the observed sensitized emission. We interpret these data to mean that only when $[PA_{63}]_7$ is present in the mixture do we observe energy transfer between labeled LF and EF, indicating both EF and LF are bound in the same $[PA_{63}]_7$ complex.

To corroborate our interpretation that co-binding of EF and LF occurs on $[PA_{63}]_7$, we formed the putative EF/LF*$[PA_{63}]_7$ complex and monitored the off-rate kinetics of EF/LF by adding a 10-fold excess of an unlabeled competitor, $LF_N$. In this case, if binding of EF and LF to $[PA_{63}]_7$ is responsible for the observed resonance energy transfer, addition of excess unlabeled competitor would be expected to displace the labeled EF and LF, resulting in loss of FRET. As shown in Fig. 2A, following addition of competitor ($LF_N$), resonance energy transfer decreased dramatically as detected by monitoring the 615 nm/525 nm fluorescence emission ratio, which is proportional to FRET efficiency. As expected for this type of interaction, exponential kinetics were observed.

Since amine-reactive chemistry was used initially to label the EF and LF proteins, some lysine side chains were labeled in addition to the N-terminus (see Materials and methods for dye/protein ratios). To control for potential perturbations due to lysine side chains being labeled and affecting $[PA_{63}]_7$ binding and observed FRET between bound EF and LF, we also prepared and labeled single-cysteine mutants of EF (G241C labeled with Alexa fluor 488 maleimide) and LF (E126C labeled with Alexa 546 maleimide) and measured sensitized emission of these proteins bound to $[PA_{63}]_7$. Fig. 2B shows the quantitative results from these experiments. These experiments demonstrate similar trends

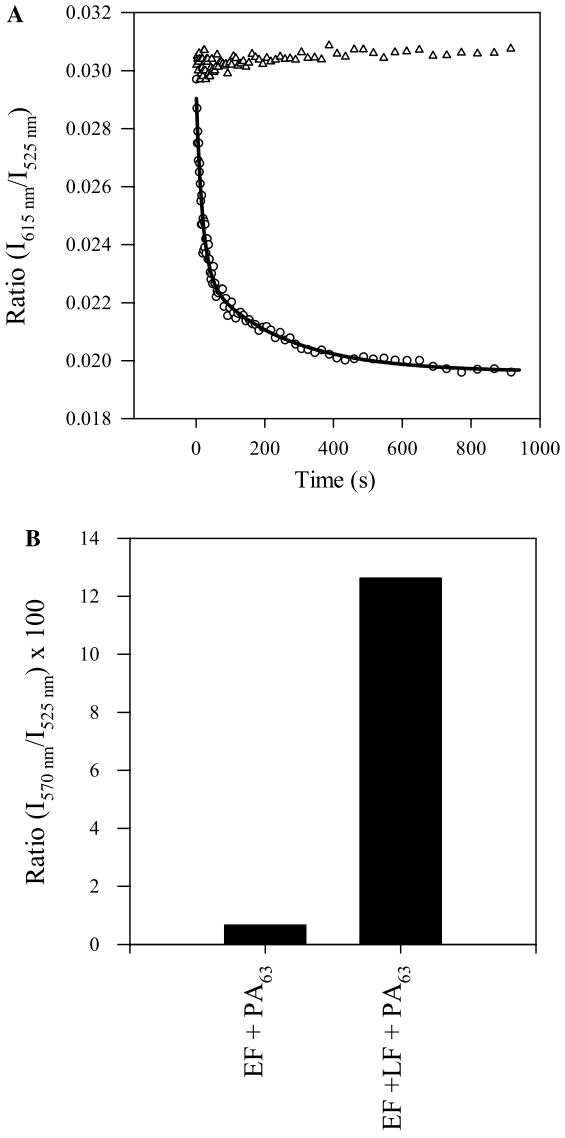


Fig. 2. (A) Kinetic transient of competition with $LF_N$. A sample containing 100 nM $[PA_{63}]_7$, 100 nM EF*488 TFP, and 230 nM LF*594 NHS was mixed with 10 μM $LF_N$ competitor at $t = 0$ and the kinetic transient was recorded (circles). As a control, buffer alone was added at $t = 0$ and the kinetic transient was recorded (triangles). (B) Ratiometric analysis of spectral data from EF G240C*488 and LF E126C*546 bound to $[PA_{63}]_7$. Ratios of the integrated intensity of the fluorescence emission at $570 \pm 10$ and $525 \pm 10$ nm were calculated for each condition. The ratio values shown have had the direct excitation of the acceptor alone subtracted prior to calculating the ratio.

in the levels of apparent FRET efficiency as observed previously. This confirms the presence of both EF and LF bound to a single molecule of $[PA_{63}]_7$ using two sets of FRET probes.

The low observed FRET efficiency (a relationship between donor–acceptor distance and the Förster distance) is due in part to the large distances between the molecules of EF and LF bound to $[PA_{63}]_7$. Based on previous mutagenesis and protein modeling of the $PA_{63}$ LF/EF interface in our laboratory, approximate distances between the labeled sites on adjacent EF and LF are predicted to be 75–85 Å [20]. If all of the donor–acceptor pairs were within this range, FRET efficiencies should be similar if not slightly greater than the values we obtained. However, the fact that various combinations of EF and LF bind to the three sites of a full occupancy $[PA_{63}]_7$ molecule under our experimental conditions dictates that there will be a distribution of donor–acceptor pair distances that will contribute to the sensitized emission signal observed. Furthermore, the apparent FRET efficiency is based on the position of the fluorophore that is attached via a 5-carbon linker and not the amino acid residues from where distances were measured. This could also reduce the observed FRET efficiencies.

The finding that EF and LF can bind concurrently to the same PA heptamer suggests that nine compositionally discrete toxic complexes could be generated by interactions of the three components of anthrax toxin: $[PA_{63}]_7$ liganded with 1, 2, or 3 molecules of either EF or LF alone, or of these proteins in 1:1, 2:1 or 1:2 ratios, respectively (Fig. 3). The degree of population of these various classes in vivo will depend on the relative concentrations of the three proteins, as well as other variables, such as temperature, that could affect the kinetics of assembly and internalization of the cell-bound complexes. It has been found that EF and LF translocate with similar efficiencies, and there is evidence that translocation efficiency is independent of the number of ligand molecules bound to $[PA_{63}]_7$ [21]. Thus, the toxicity brought about by a molecule of EF or LF is unlikely to be affected by another ligand molecule or molecules bound to the same $PA_{63}$ heptamer.

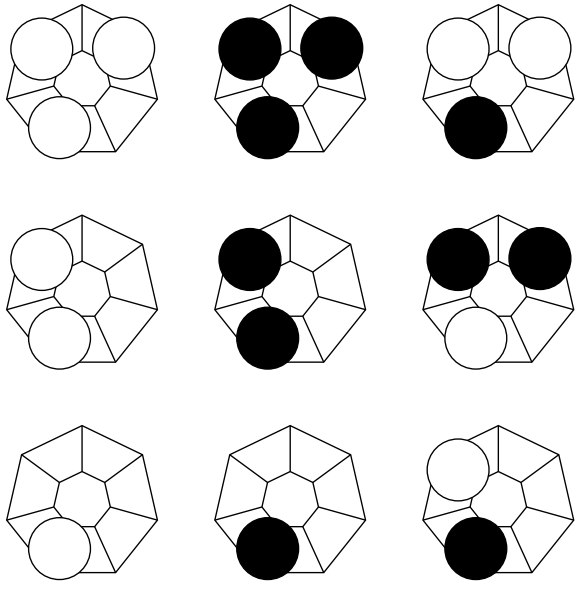

Fig. 3. Schematic representation of possible discrete anthrax toxin complexes. EF is represented by open circles. LF is represented by solid circles. Wedges represent $PA_{63}$ in oligomeric form $[PA_{63}]_7$.

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## References

[1] H. Smith, H.B. Stoner, Anthrax toxic complex, Fed. Proc. 26 (1967) 1554–1557.
[2] H. Smith, Discovery of the anthrax toxin: the beginning of in vivo studies on pathogenic bacteria, Trends Microbiol. 8 (2000) 199–200.
[3] N.S. Duesbery, J. Resau, C.P. Webb, S. Koochekpour, H.M. Koo, S.H. Leppla, G.F. Vande Woude, Suppression of ras-mediated transformation and inhibition of tumor growth and angiogenesis by anthrax lethal factor, a proteolytic inhibitor of multiple MEK pathways, Proc. Natl. Acad. Sci. USA 98 (2001) 4089–4094.
[4] R. Pellizzari, C. Guidi-Rontani, G. Vitale, M. Mock, C. Montecucco, Lethal factor of *Bacillus anthracis* cleaves the N-terminus of MAPKKs: analysis of the intracellular consequences in macrophages, Int. J. Med. Microbiol. 290 (2000) 421–427.
[5] G. Vitale, R. Pellizzari, C. Recchi, G. Napolitani, M. Mock, C. Montecucco, Anthrax lethal factor cleaves the N-terminus of MAPKKs and induces tyrosine/threonine phosphorylation of MAPKs in cultured macrophages, Biochem. Biophys. Res. Commun. 248 (1998) 706–711.
[6] K.A. Bradley, J. Mogridge, M. Mourez, R.J. Collier, J.A. Young, Identification of the cellular receptor for anthrax toxin [comment], Nature 414 (2001) 225–229.
[7] H.M. Scobie, G.J. Rainey, K.A. Bradley, J.A. Young, Human capillary morphogenesis protein 2 functions as an anthrax toxin receptor, Proc. Natl. Acad. Sci. USA 100 (2003) 5170–5174.
[8] K.R. Klimpel, S.S. Molloy, G. Thomas, S.H. Leppla, Anthrax toxin protective antigen is activated by a cell surface protease with the sequence specificity and catalytic properties of furin, Proc. Natl. Acad. Sci. USA 89 (1992) 10277–10281.
[9] S.S. Molloy, P.A. Bresnahan, S.H. Leppla, K.R. Klimpel, G. Thomas, Human furin is a calcium-dependent serine endoprotease that recognizes the sequence Arg-X-X-Arg and efficiently cleaves anthrax toxin protective antigen, J. Biol. Chem. 267 (1992) 16396–16402.
[10] C. Petosa, R.J. Collier, K.R. Klimpel, S.H. Leppla, R.C. Liddington, Crystal structure of the anthrax toxin protective antigen, Nature 385 (1997) 833–838.
[11] J.L. Elliott, J. Mogridge, R.J. Collier, A quantitative study of the interactions of *Bacillus anthracis* edema factor and lethal factor with activated protective antigen, Biochemistry 39 (2000) 6706–6713.
[12] J. Mogridge, K. Cunningham, R.J. Collier, Stoichiometry of anthrax toxin complexes, Biochemistry 41 (2002) 1079–1082.
[13] L. Abrami, S. Liu, P. Cosson, S.H. Leppla, F.G. van der Goot, Anthrax toxin triggers endocytosis of its receptor via a lipid raft-mediated clathrin-dependent process [comment], J. Cell Biol. 160 (2003) 321–328.
[14] J.C. Milne, R.J. Collier, pH-dependent permeabilization of the plasma membrane of mammalian cells by anthrax protective antigen, Mol. Microbiol. 10 (1993) 647–653.
[15] R.O. Blaustein, T.M. Koehler, R.J. Collier, A. Finkelstein, Anthrax toxin: channel-forming activity of protective antigen in planar phospholipid bilayers, Proc. Natl Acad. Sci. USA 86 (1989) 2209–2213.
[16] J. O'Brien, A. Friedlander, T. Dreier, J. Ezzell, S. Leppla, Effects of anthrax toxin components on human neutrophils, Infect. Immun. 47 (1985) 306–310.
[17] D.J. Wigelsworth, B.A. Krantz, K.A. Christensen, D.B. Lacy, S.J. Juris, R.J. Collier, Binding stoichiometry and kinetics of the interaction of a human anthrax toxin receptor, CMG2, with protective antigen, J. Biol. Chem. 279 (2004) published online.
[18] A. Barnard, M. Payton, Fermentation and growth of Escherichia coli for optimal protein production, in: J.E. Coligan, B.M. Dunn, H.L. Plough, D.W. Speicher, P.T. Wingfield (Eds.), Current Protocols in Protein Science, Vol. Chap. 5.3, Wiley, New York, 1995, pp. 1–18.
[19] J.R. Lakowicz, Principles of Fluorescence Spectroscopy, second ed., Kluwer Academic/Plenum Publishers, New York, 1999.
[20] D.B. Lacy, K.A. Christensen, unpublished results.
[21] S. Zhang, K. Cunningham, R.J. Collier, Anthrax protective antigen: efficiency of translocation is independent of the number of ligands bound to the prepore, Biochemistry 43 (2004) 6339–6343.