Fluorescence resonance energy transfer studies on anthrax lethal toxin

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Abstract **Anthrax lethal toxin is a binary bacterial toxin consisting of two proteins, protective antigen (PA) and lethal factor (LF), that self-assemble on receptor-bearing eukaryotic cells to form toxic, non-covalent complexes. PA_{63}, a proteolytically activated form of PA, spontaneously oligomerizes to form ring-shaped heptamers that bind LF and translocate it into the cell. Site-directed mutagenesis was used to substitute cysteine for each of three residues (N209, E614 and E733) at various levels on the lateral face of the PA_{63} heptamer and for one residue (E126) on LF_N, the 30 kDa N-terminal PA binding domain of LF. Cysteine residues in PA were labeled with IAEDANS and that in LF_N was labeled with Alexa 488 maleimide. The mutagenesis and labeling did not significantly affect function. Time-resolved fluorescence methods were used to study fluorescence resonance energy transfer (FRET) between the AEDANS and Alexa 488 probes after the complex assembled in solution. The results clearly indicate energy transfer between AEDANS labeled at residue N209C on PA and the Alexa 488-labeled LF_N, whereas transfer from residue E614C on PA was slight, and none was observed from residue E733C. These results support a model in which LF_N binds near the top of the ring-shaped $(PA_{63})_7$ heptamer.**

Key words: Anthrax; Lethal factor; Fluorescence; Lifetime; Fluorescence resonance energy transfer

1. Introduction

Anthrax toxin, secreted by *Bacillus anthracis*, the causative agent of anthrax, consists of an 83 kDa receptor binding protein termed protective antigen (PA) and two enzymatic proteins termed edema factor (EF; 89 kDa) and lethal factor (LF; 83 kDa). These proteins are released from the bacteria as non-toxic monomers and act in binary or ternary combinations to elicit toxic responses. The combination of PA and LF alone can cause death and has been termed lethal toxin.

The 83 kDa PA diffuses to the cell surface, binds to the ATR receptor [1] and is cleaved into two fragments by a furin protease. The N-terminal 20 kDa fragment dissociates and diffuses away from the cell surface [2], while the 63 kDa fragment (PA_{63}) remains associated with the receptor and assembles on the cell surface into a symmetric, ring-shaped heptameric structure [3,4]. This heptamer is the precursor of a pore, and has been termed the prepore. It is also able to competitively bind the enzymatic factors, EF or LF, with high affinity ($K_d \sim 1$ nM) [5–7]. The resulting complexes are endocytosed into the cell and trafficked to a low-pH compartment [8]. There, they undergo a pH-dependent conformational change [5] that leads to membrane insertion by the PA_{63} heptamer and translocation of EF or LF to the cytosol. EF is then able to bind calmodulin and catalyze the conversion of adenosine triphosphate (ATP) into cAMP, while LF – a zinc-dependent protease – can cleave certain mitogen-activated protein kinase kinases leading to macrophage death.

Although the three-dimensional structures of both the heptameric prepore and LF have appeared [9–12], the spatial relationships between the PA_{63} moieties and the LF (or EF) molecules in the toxic complexes remain open to question. Recent studies utilizing isotopic labeling and light-scattering suggest that the PA heptamer binds up to three molecules of LF and/or EF under saturating conditions [13]. Additional studies on an oligomerization-deficient mutant of PA_{63} demonstrated that EF and LF bind only to dimers or higher order oligomers of PA_{63} [14]. Since removal of the PA_{20} fragment is required before the enzymatic proteins can bind, it was hypothesized that the newly exposed surface on the amino-terminal region of PA_{63} (domain 1′), is involved in the binding process [9]. This hypothesis was supported by studies showing that substitution of alanine for any of seven residues in or near the domain 1′ region of PA_{63} strongly inhibited LF or EF binding [15].

The present study was undertaken to provide spectroscopic information on the spatial arrangements of ligands such as LF in the complex with the PA_{63} heptamer. Ultimately, the fluorescent labeling and characterization of this system will also allow for more detailed solution studies of the conformational dynamics in this system.

2. Materials and methods

2.1. Construction of protein mutations

Cysteine residues were introduced in PA_{83} at positions N209, E614 and E733, and at position E126C in LF_N, using the methodologies previously described [15]. The recombinant proteins were then purified from *Escherichia coli*, as previously described [4].

2.2. Labeling of cysteine residues with thio-reactive fluorescence probes

The PA_{83} and LF_N cysteine mutants were labeled, respectively, with IAEDANS (Molecular Probes, Eugene, OR, USA) and Alexa Fluor 488 C_5 maleimide (Molecular Probes, Eugene, OR, USA) using methodologies previously described [16].

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2.3. Activation of PA and oligomer formation

PA_{83} was catalytically activated and then prepore was prepared as previously described [15].

2.4. Cell surface binding assay

The cell surface assay was used to measure the PA-mediated binding of ^{35}S-labeled LF_N and was carried out as previously described [15]. Briefly, CHO-K1 cells were incubated on ice with 2.4×10^{-8} M activated PA for 2 h. The cells were washed twice with cold Dulbecco's phosphate-buffered saline (PBS) and then incubated on ice with [^{35}S]LF_N for 2 h. The [^{35}S]LF_N was produced by in vitro transcription/translation. The cells were washed three times with cold Dulbecco's PBS and then lysed. Radioactive content was determined by scintillation counting. % Binding = (cpm [^{35}S]LF_N bound by mutant/cpm [^{35}S]LF_N bound by wild-type PA).

2.5. Inhibition of protein synthesis

Inhibition of protein synthesis was used to measure the ability of PA to translocate an LF_N-DTA ligand into cytosol [17]. LF_N-DTA is a fusion protein containing LF_N and DTA, the enzymatic domain of diphtheria toxin. Delivery of LF_N-DTA into the cell cytosol allows the DTA moiety to catalyze the adenosine diphosphate (ADP) ribosylation of elongation factor 2, thereby inhibiting protein synthesis. PA (1×10^{-12}–1×10^{-7} M) was incubated with CHO-K1 cells in the presence of 1×10^{-10} M LF_N-DTA for 4 h at 37°C. The medium was replaced with leucine-free medium supplemented with [^{3}H]leucine at 1 μCi/ml for 1 h at 37°C. Cells were washed with Dulbecco's PBS and proteins precipitated with 10% trichloroacetic acid. The precipitated proteins were resuspended first with 0.2 M KOH, followed by an equal volume of 0.1 M HCl. The amount of titrated proteins was determined by scintillation counting. Cells incubated with LF_N-DTA in the absence of PA were used as standard for 100% protein synthesis.

2.6. Fluorescence spectroscopy

Time-resolved fluorescence measurements were performed using an ISS K2 multifrequency phase and modulation spectrofluorometer (ISS Inc., Champaign, IL, USA) equipped with a Spectra-Physics model 2045 Argon ion laser (Spectra-Physics, Mountain View, CA, USA) as the excitation source. Samples were excited using the 351 nm argon ion line. Emission was observed using a combination of a Schott KV399 cut-on filter and Andover 500 (FL 07-50) cut-off filter; this filter combination passes the wavelength range of 380–505 nm (these wavelengths represent 5% transmittance levels), which isolates the AEDANS emission from the Alexa emission. Dimethylpopop in ethanol was used as a lifetime reference standard ($\tau = 1.41$ ns). Lifetime data were analyzed with software provided by either ISS or Globals Unlimited, using previously described fitting routines [18–20]. Data analysis assumed standard errors of 0.2° and 0.004 for phase and modulation values, respectively.

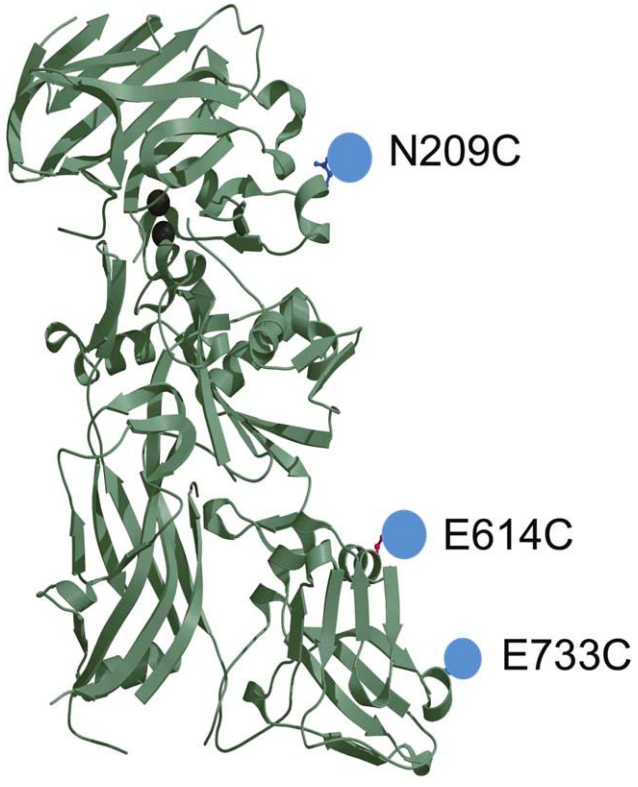

Fig. 1. Structure of PA (PA_{83}) with positions of the cysteine mutations indicated (N209C, E614C, E733C) adapted from [9].

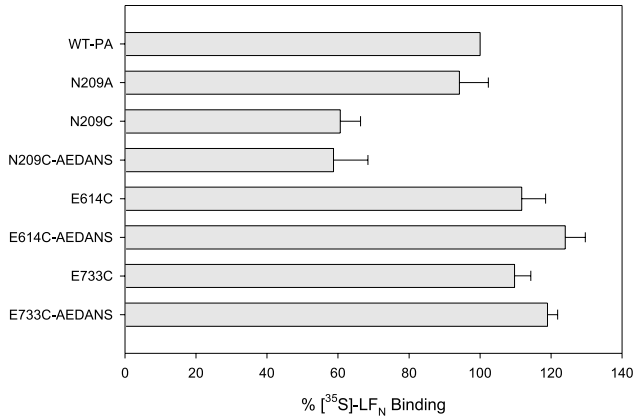

Fig. 2. Cell binding assay results for labeled and unlabeled PA proteins. Percent binding of [^{35}S]LF_N is relative to wild-type PA (WT-PA).

3. Results and discussion

The three-dimensional structure of the PA_{83} species shown in Fig. 1 illustrates the locations of the three residues (N209, E614, and E733), that were replaced by cysteines. The residues are on the outer lateral surface of the final heptameric prepore assembly and were chosen to localize probes near the top (N209), middle (E614) and lower (E733) sections of this surface.

Each mutant protein was tested for functional competence using a cell binding assay and a diphtheria toxin linked protein synthesis inhibition assay. The results of the cell binding assay are shown in Fig. 2. In all cases, LF_N was able to bind

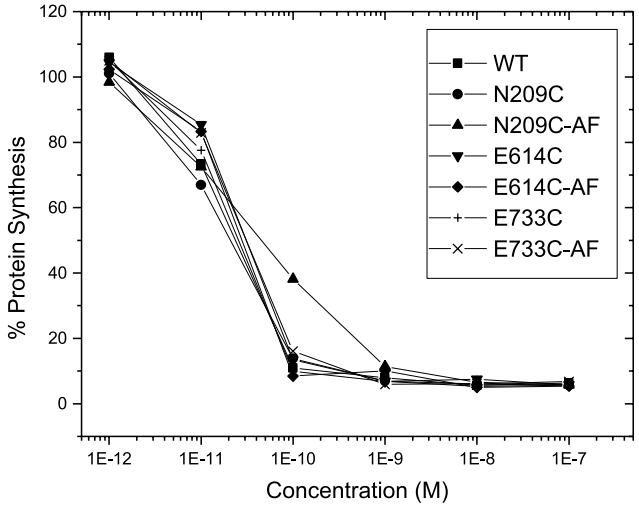

Fig. 3. Results of protein synthesis inhibition assay for labeled and unlabeled PA proteins.

to the fluorophore-labeled mutant PA proteins, which were bound to the cells, and in some cases the binding increased relative to wild-type PA. The results of the diphtheria toxin linked protein inhibition assay for the wild-type and mutant/labeled proteins are shown in Fig. 3. In no case did the cysteine mutation and subsequent IAEDANS labeling of PA lead to significant loss of activity.

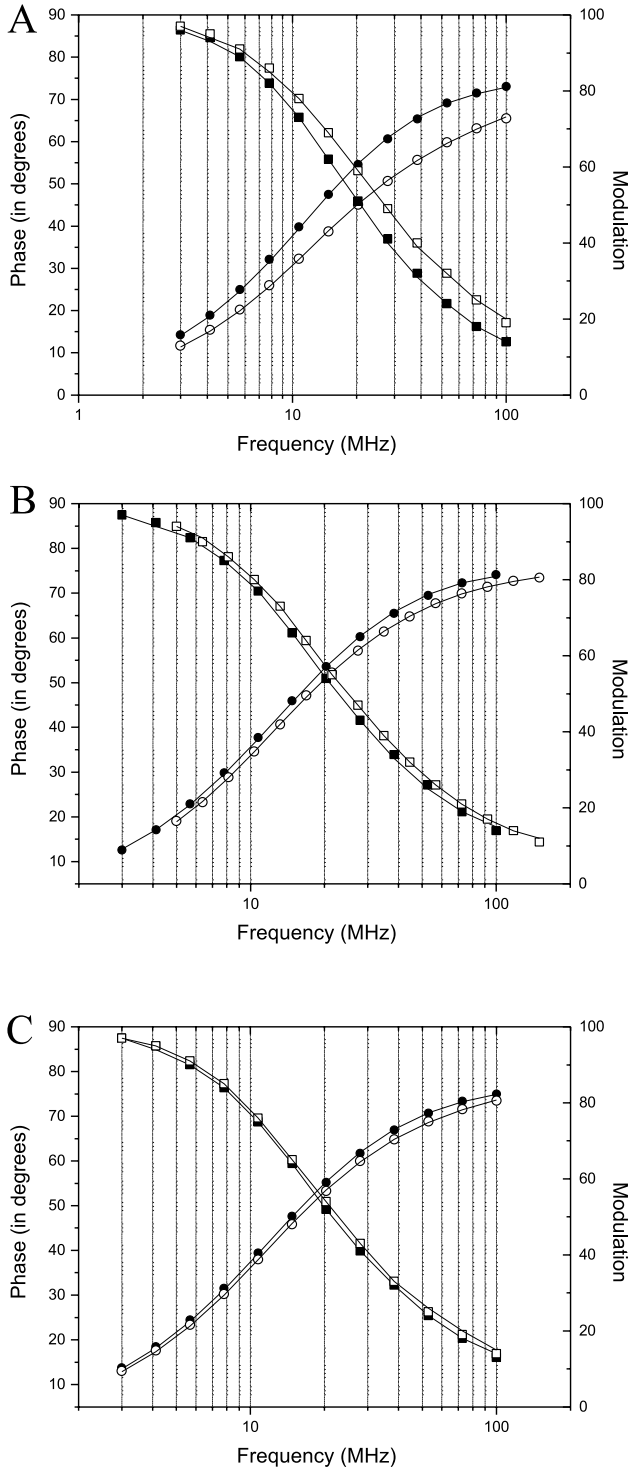

Fig. 4. Multifrequency phase (circles) and modulation (squares) data for AEDANS covalently attached to the cysteine residues of three PA mutant proteins: A: N209C, B: E614C, C: E733C assembled into the prepore. Data are presented for prepore free (solid symbols) and associated with Alexa-LF_N (open symbols).

Table 1
AEDANS lifetime in the absence and presence of Alexa-LF_N

AEDANS location on PA_{63}	AEDANS major lifetime (ns) component (center value of Lorentzian distribution)[a]	
	$-LF_N$	$+LF_N$
N209C	13.2	11.8
E614C	12.0	11.8
E733C	12.8	12.8

[a]The 67% confidence limits of these major lifetime components had less than 2% associated errors.

The fluorescence lifetime of AEDANS was measured, using the multifrequency phase and modulation technique, for the case of the three prepore mutants alone or complexed with Alexa-LF_N. Fig. 4 displays these phase and modulation data. As indicated in Table 1, the AEDANS lifetimes for the PA heptamers in the absence of Alexa-LF_N varied with the labeling position, apparently reflecting the different environments around the probe. The data were analyzed using a three-component model and the results, shown in Table 1, indicate that efficient energy transfer only occurred between AEDANS and Alexa-LF_N for AEDANS on position N209C on the prepore. In all cases the lifetime data fit best to three components, specifically two minor components (a short (<1 ns) component and a fixed 3.5 ns component included to account for a small fraction of the Alexa emission which leaked through the filter combination) and a major, longer component described as a Lorentzian distribution [18]. The critical transfer distance (R_0) for the AEDANS/Alexa 488 donor/acceptor pair is in the range of 40–50 Å (since the absorption and emission of Alexa 488 closely resemble that of fluorescein), depending upon the actual absorption and emission maxima of the probes in their particular local environments [21]; since the emission spectrum of AEDANS is slightly environmentally sensitive and changes depending on the labeling site, the R_0 value will also change for the different sites. However, given the fact that our system contains seven AEDANS probes on the prepore and that up to three Alexa-LF_N molecules may bind to this prepore, precise distance determinations are not possible, at any rate from the fluorescence resonance energy transfer (FRET) data. But the results clearly indicate that the efficiency of energy transfer decreases as the donor probe (AEDANS) is positioned further down the lateral face of the prepore, i.e. further away from the prepore top surface, supporting previous studies indicating that LF binds to the top surface of the prepore [15]. More detailed FRET studies on this system should be valuable in revealing the precise spatial relationships between the proteins in this complex assembly.

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