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Effect of pH on the catalytic function and zinc content of native and immobilized anthrax lethal factor

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ABSTRACT

Translocation of the zinc-dependent metalloendopeptidase anthrax lethal factor (LF) from the endosome to the cytosol requires an acidic endosomal milieu. In the current study, we utilized immobilized (to prevent protein aggregation below pH 5.5) and native LF to assess the effect of pH on the function and metal content of LF. Our results reveal the diminution of LF's catalytic competence under moderately acidic conditions (pH ~6) to be uncorrelated to the metal content of the protein. However, a significant degree of demetallation of LF (~30%) was observed at pH values close to those found in late endosomes (pH ~5), thus raising the possibility that a substantial proportion of LF molecules may not be in their zinc-bound state prior to translocation.

Structured summary of protein interactions:
LF and **LF** bind by fluorescence technology (View interaction)

1. Introduction

Anthrax toxin (AT), an exotoxin secreted by *Bacillus anthracis* and a major virulence factor of anthrax, is a tripartite protein comprised of protective antigen (PA), edema factor (EF) and lethal factor (LF) [1]. While EF acts as an adenylyl cyclase responsible for the increase in intracellular cAMP levels [2], LF is a zinc-dependent metalloendopeptidase involved in the cleavage of mitogen-activated protein kinase kinases near their N-termini [3–6], and thus in the impairment of critical intracellular signaling pathways [7]. In addition, LF has recently been implicated in catalyzing the removal of the N-terminal segment of NOD-like receptor protein 1 (Nlrp1), leading to inflammasome activation and macrophage death [8]. PA (in its oligomeric form) is a pore-forming protein which mediates the entry of LF and EF from the endosome into the host cell cytosol [9–11]. In view of the dimensions of the central lumen of the PA pore (~15 Å diameter at its narrowest point), it has been proposed that structural arrangements only as large as a single α-helix are capable of passing through the pore [12,13]. Consequently, PA-assisted translocation of LF and EF requires the (at least) partial unfolding of the enzymatic components [12–15]. In addition, an acidic milieu, imposed by the vacuolar H^+-ATPases in the endosomal membrane [16], is stringently required to not only trigger pore formation, but also to assist in the initiation of unfolding of the N-terminus of LF (or EF) [15]. Given that the acidification of zinc proteins can render the metal ion prone to dissociation (by virtue of protonation of the metal-binding ligands [17]), it is not inconceivable that LF's Zn^{2+} ion (being bound to His686, His690 and Glu735) is released at low endosomal pH. However, whether LF's Zn^{2+}-binding motif can stay intact under these conditions remains to be established.

Previous studies on the pH dependence of LF's catalytic competence have revealed the enzymatic activity of the protein to be significantly reduced below pH 6, and to be almost completely abolished at pH 4 [18,19]. Although the molecular basis underlying these observations has not been elucidated, loss of the catalytically essential metal ion might provide a plausible explanation for this phenomenon.

The current investigations were aimed at elucidating the relationship between LF's catalytic function and its metal status at pH values encountered within the endosome. It is important to note that preliminary studies on the influence of pH on LF revealed the protein to precipitate below pH 5.5, a feature which could potentially affect both the pH-dependence of catalytic function and the enzyme's ability to bind metal ions. To avoid aggregation,

Abbreviations: AMT, acetic acid/MES/Tris; AT, anthrax toxin; cAMP, adenosine 3′,5′-cyclic monophosphate; EF, edema factor; I_c, ionic strength (concentrational); $LF_{(nat)}$, (native) lethal factor; LF_{im}, immobilized lethal factor; MES, 2-(*N*-morpholino)ethanesulfonic acid; NHS, *N*-hydroxysuccinimide; PA, protective antigen; *S*-*p*NA, lethal factor substrate; Tris, tris(hydroxymethyl)aminomethane

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LF was immobilized onto *N*-hydroxysuccinimide (NHS) activated agarose beads (see Fig. 1), and its catalytic competence and metal content was assessed as a function of pH, with the non-immobilized, native form of the protein serving as a reference.

2. Materials and methods

2.1. General

Lethal factor protease substrate II (*S*-*p*NA) was purchased from EMD Biosciences (La Jolla, CA). All other chemicals were of analytical grade and obtained from Sigma–Aldrich (St. Louis, MO). All solutions were prepared using MilliQ ultrapure water ($\geqslant$18.2 MΩ cm resistivity). Unless stated otherwise, AMT, a three-component buffer consisting of acetic acid, 2-(*N*-morpholino)ethanesulfonic acid (MES) and tris(hydroxymethyl)aminomethane (Tris) in a 1:1:2 molar ratio was utilized in the current studies to maintain a constant ionic strength (I_c) over the entire pH range investigated [20]. The pH of the AMT buffer was adjusted to the desired value using either HCl or NaOH.

2.2. Isolation and immobilization of LF

LF was isolated from *Bacillus megaterium* harboring the pWH1520-LF plasmid (MoBiTec, Göttingen, Germany) and purified as described previously [21]. The Zn^{2+} content of purified LF was determined with 4-(2-pyridylazo)resorcinol as outlined earlier [22], and was found to be 1.0 (±0.1) Zn^{2+} per protein molecule. Immobilization of LF was achieved by covalently coupling the protein to *N*-hydroxysuccinimide (NHS)-activated agarose beads (Pierce, Rockford, IL) according to the manufacturer's instructions, using spin columns containing either 33 or 330 mg of resin. In brief, LF was exchanged into sodium phosphate buffer (100 mM, pH 7.2) containing 0.15 M NaCl prior to coupling. Immobilization of LF was achieved by incubating the protein (10 μM) in sodium phosphate buffer in the presence of the beads for 1 h at room temperature. Following coupling, the mixture was centrifuged at 1000×*g* for 1 min, and the flow-through was kept for the determination of the protein coupling efficiency (see below). The beads were then washed twice with sodium phosphate buffer. Any unreacted (NHS-containing) sites were subsequently blocked by treatment with ethanolamine (1 M, pH 7.4) for 30 min at room temperature. Following centrifugation, the beads were washed three times with AMT buffer (12.5 mM acetic acid, 12.5 mM MES and 25 mM Tris; I_c = 0.025; pH 7.0) to remove excess ethanolamine. The protein coupling efficiency was determined using the Pierce 660 nm protein assay according to manufacturer's instructions.

2.3. Determination of enzymatic activity

To maintain a constant ionic strength over the pH range investigated (pH 4.0–8.0), AMT buffer (I_c = 0.025) was utilized as the medium in the assessment of the catalytic competence of LF. The choice of the ionic strength (I_c = 0.025) was based on the observation that LF is stable and catalytically fully functional under these conditions [19]. Following incubation of the protein at the desired pH for 3 min at room temperature, the enzymatic activity of the native form of LF (LF_{nat}) was determined spectrophotometrically at 405 nm using *S*-*p*NA as reported in the literature [22,23]. In the case of immobilized LF (LF_{im}), the protocol for the determination of the enzyme's catalytic competence required significant alterations in view of increased light scattering at 405 nm, and the occurrence of incomplete mixing of substrate and enzyme as a consequence of the agarose beads settling rapidly to the bottom of the spectrophotometer cell. Hence, the activity of LF_{im} was assessed by exposing the protein (100 nM) to AMT buffer (I_c = 0.025) of the desired pH for 3 min at room temperature prior to the initiation of the reaction by the addition of *S*-*p*NA (10 μM). Following exposure to the substrate for 120 s, LF_{im} was removed rapidly (within 5 s) by passing the mixture through an empty spin column. The concentration of the reaction product (*p*-nitroaniline) was determined at 405 nm using an extinction coefficient of 9920 M^{-1} cm^{-1} [23].

2.4. Assessment of zinc release from LF

In the case of LF_{nat}, a stock solution of the enzyme (35 μM) was diluted with AMT buffer (I_c = 0.0188) of the desired pH to achieve a final concentration of 0.6 μM with respect to the enzyme. Following incubation for 3 min at room temperature, the protein was removed by centrifugation using an Amicon Ultra centrifugal concentrator (30 kDa molecular weight cut-off; Millipore, Bedford, MA), and the recovered filtrate was diluted threefold with MilliQ water prior to metal determination. In case of complete release of Zn^{2+} from LF, the expected concentration of the ion in the filtrate was 0.2 μM (13.1 ppb).

To assess the pH-dependence of Zn^{2+} dissociation from LF_{im}, 100 μl of the enzyme ([LF_{im}] = 6.8 μM in AMT buffer [I_c = 0.025, pH = 7.0]) was supplemented with an equal volume of AMT buffer (I_c = 0.1) to achieve the desired pH. Following incubation for 3 min at room temperature, the mixture was passed through an empty spin column to remove the immobilized protein. The filtrate was subsequently diluted 10-fold with MilliQ water so as to achieve a final ionic strength of AMT buffer identical to that outlined above for LF_{nat} (i.e., I_c = 0.00625). In case of complete release of Zn^{2+} from LF, the anticipated concentration of the ion in the filtrate was 0.34 μM (22.2 ppb).

The amount of Zn^{2+} in the filtrates was determined by inductively-coupled plasma mass spectrometry using a Perkin Elmer Sciex Elan 6000 mass spectrometer (Waltham, MA). External Zn^{2+} standards (1–40 ppb) were prepared in AMT buffer of the same ionic strength as that used for protein samples (i.e., I_c = 0.00625). All samples (including standards) were supplemented with HNO_3 (trace metal grade) to achieve a final concentration of 1% (w/v) prior to measurement (in triplicate) using standard operating conditions.

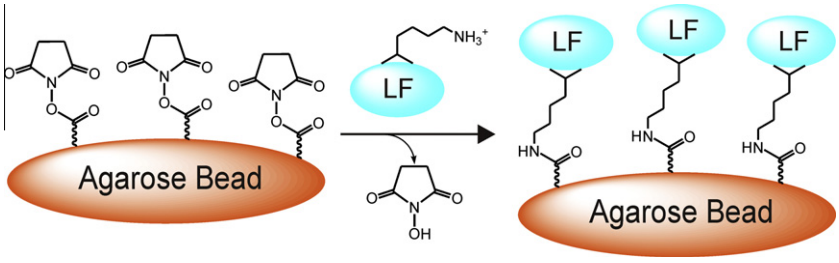

Fig. 1. Schematic representation of the immobilization of LF using NHS-activated agarose beads.