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Influence of chemical denaturants on the activity, fold and zinc status of anthrax lethal factor

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Anthrax lethal factor (LF) is a zinc-dependent endopeptidase which, through a process facilitated by protective antigen, translocates to the host cell cytosol in a partially unfolded state. In the current report, the influence of urea and guanidine hydrochloride (GdnHCl) on LF's catalytic function, fold and metal binding was assessed at neutral pH. Both urea and GdnHCl were found to inhibit LF prior to the onset of unfolding, with the inhibition by the latter denaturant being a consequence of its ionic strength. With the exception of demetallated LF (apoLF) in urea, unfolding, as monitored by tryptophan fluorescence spectroscopy, was found to follow a two-state (native to unfolded) mechanism. Analysis of the metal status of LF with 4-(2-pyridylazoresorcinol) (PAR) following urea or GdnHCl exposure suggests the enzyme to be capable of maintaining its metal ion passed the observed unfolding transition in a chelator-inaccessible form. Although an increase in the concentration of the denaturants eventually allowed the chelator access to the protein's zinc ion, such process is not correlated with the release of the metal ion. Indeed, significant dissociation of the zinc ion from LF was not observed even at 6 M urea, and only high concentrations of GdnHCl (> 3 M) were capable of inducing the release of the metal ion from the protein. Hence, the current study demonstrates not only the propensity of LF to tightly bind its zinc ion beyond the spectroscopically determined unfolding transition, but also the utility of PAR as a structural probe.

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1. Introduction

Anthrax lethal factor (LF) is a zinc-dependent metalloendopeptidase and, along with protective antigen (PA) and edema factor (EF), one of the three protein components constituting the anthrax toxin [1–3]. While PA is quintessential for the delivery of LF and EF to the host cell cytosol *via* the formation of a heptameric or octameric pore in the membrane of the acidified endosomal compartment [4–7], EF has been shown to be a calcium and calmodulin-dependent adenylyl cyclase involved in the dysregulation of water homeostasis by catalyzing the production of excessive amounts of cyclic AMP [8].

LF belongs to the family of gluzincins and exerts its deleterious intracellular action by removing N-terminal segments from most members of the mitogen-activated protein kinase kinase (MAPKK) family of signaling proteins [9,10], and from NOD-like receptor protein 1 (Nlrp1) resulting in the activation of the inflammasome and macrophage death [11–13]. The Zn^{2+} ion in the active site of LF is bound tetrahedrally to the side chains of His686, His690 and Glu735, and to the nucleophilic water molecule responsible for the cleavage of the scissile peptide bond in the substrates [14]. In addition, Glu687, which is part of the thermolysin-like HExxH consensus motif [15], functions as the general base in the catalytic mechanism of LF [14].

As outlined above, PA (in its oligomeric, pore-forming state) mediates the translocation of LF and EF from the endosome to the cytosol. In view of the constriction (to ~15 Å) of the central lumen of the pore, it appears unlikely that secondary structural motifs larger than a single α-helix are capable of migrating through the pore [16,17]. Hence, the translocation of LF and EF through the PA channel necessitates the partial, pH-assisted unfolding of these enzymatic entities [17–19]. Whether LF's Zn^{2+} ion remains associated to the protein during PA-mediated translocation is currently unknown. While it has recently been shown that acidification of LF to pH 5 (pH conditions encountered in late endosomes) leads to

Abbreviations: CD, circular dichroism; cps, counts per second; DPA, dipicolinic acid; EDTA, ethylenediaminetetraacetic acid; EF, edema factor; LF, anthrax lethal factor; MWCO, molecular weight cut-off; PA, protective antigen; PAR, 4-(2-pyridylazo)resorcinol; *S*-*p*NA, lethal factor substrate; SASA, solvent-accessible surface area; SOD, superoxide dismutase; ZnLF, zinc-containing lethal factor

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partial (~30%) demetallation of the enzyme active site [20], the effect of unfolding on the integrity of the protein's Zn^{2+}-binding motif has not been investigated previously.

The current studies were designed to elucidate the effect of ionic and non-ionic denaturants (guanidine hydrochloride and urea, respectively) on the catalytic competence and fold of full-length LF, as well as their influence on the binding of the active site Zn^{2+} ion at neutral pH. Structural perturbations as a consequence of denaturant exposure were investigated by intrinsic tryptophan fluorescence spectroscopy, and revealed the protein to typically unfold *via* a two-state (native to unfolded) mechanism. Furthermore, the analysis of the metal status of LF following supplementation of the protein with urea/GdnHCl at moderate concentrations suggests the enzyme to be capable of retaining its metal ion passed the spectroscopically observed unfolding transition in a chelator-inaccessible form. The actual dissociation of the Zn^{2+} ion from the protein appears to require much higher concentrations of the chaotropes, hence, highlighting LF's propensity to tightly bind its metal ion even when in a denatured state.

2. Materials and methods

2.1. General

Chromogenic anthrax lethal factor protease substrate II, *S*-*p*NA (Ac-GYβARRRRRRRRVLR-*p*NA, *p*NA=*para*-nitroanilide) was obtained from Biomatik (Cambridge, ON, Canada). Urea, guanidine hydrochloride (GdnHCl) and guanidine isothiocyanate (GdnSCN) were purchased from BioShop (Burlington, ON, Canada), and were of the highest available purity. All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO). All solutions were prepared using MilliQ ultrapure water ($\geq$ 18.2 MΩ cm resistivity).

2.2. Isolation of LF

Wild-type LF (ZnLF) was expressed in *Bacillus megaterium* and purified according to published procedures [21]. The Zn^{2+} content of LF was assessed using the chromophoric chelator 4-(2-pyridylazo)resorcinol (PAR) as outlined previously [22], and was found to be 0.95 ($\pm$0.10) Zn^{2+}/protein molecule. ApoLF with a residual Zn^{2+} content of less than 0.03 Zn^{2+}/LF molecule was obtained by demetallation of ZnLF with the aid of dipicolinic acid (DPA) and ethylenediaminetetraacetic acid (EDTA) as documented recently [23].

2.3. Enzymatic assays

The enzymatic activity of LF preparations was assessed using the chromogenic *S*-*p*NA substrate according to published procedures [22,24]. In a typical assay (final volume: 0.1 mL), LF (50 nM) in Hepes buffer (50 mM, pH 7.4) as allowed to equilibrate at room temperature for 1 min prior to the initiation of the reaction by the addition of substrate (final concentration of 10 μM). The progress of substrate hydrolysis was monitored at 405 nm using a Cary 60 UV–vis spectrophotometer (Agilent, Santa Clara, CA). The half-inhibitory concentrations (IC_{50} values) of $MgSO_4$, Gdn-containing salts as well as their sodium analogs were determined in a fashion analogous to that outlined above, except for the incubation of the enzyme in the presence of these salts for 30 min prior to the supplementation of the assay with *S*-*p*NA.

2.4. Accessibility of Zn^{2+} to chelation by PAR

The accessibility of LF-bound Zn^{2+} to chelation by PAR as a function of denaturant concentration was assessed in a 96-well microplate format. In a volume of 190 μL (per well), ZnLF was exposed to GdnHCl or urea at various concentrations in Hepes buffer (50 mM, pH 7.4) for 1 h and 24 h at room temperature prior to the addition of 10 μL PAR (1 mM) to yield final concentrations of 5 μM and 50 μM with respect to the enzyme and chelator, respectively. Following supplementation with PAR, the progress of the reaction of the chelator with LF's Zn^{2+} ion was monitored spectrophotometrically at 500 nm for 60 min (10 s intervals) using a BioTek Epoch microplate spectrophotometer (Winooski, VT). Standards containing $ZnSO_4$ (0–6 μM), denaturant (at concentrations identical to those of the LF samples) and PAR (50 μM) were prepared and measured under analogous conditions alongside the protein samples. The degree of complexation of LF's Zn^{2+} ion by PAR was determined based on the linear relationship between the concentration of Zn^{2+} in the standard and the absorbance at 500 nm (assessed after each 10 s time interval).

2.5. Metal release studies

The release of Zn^{2+} from LF as a function of the denaturant concentration was determined with the aid of PAR as follows: In a final volume of 550 μL, LF (5 μM) in Hepes buffer (50 mM, pH 7.4) was exposed to urea or GdnHCl at the desired concentration for 1 h at room temperature. Following incubation, a 275 μL aliquot was withdrawn from the mixture and immediately subjected to filtration using a 0.5 mL Amicon Ultra-15 centrifugal filter (30 kDa MWCO; Millipore, Bedford, MA). The remaining sample (275 μL) was incubated for another 23 h prior to Amicon filtration. The concentration of released Zn^{2+} was assessed spectrophotometrically by incubating 190 μL of each filtrate with 10 μL of PAR (1 mM) for 1 h, followed by monitoring of the absorbance of the PAR-Zn^{2+} complex at 500 nm. Standards containing $ZnSO_4$ (0–6 μM), denaturant (at the appropriate concentration) and PAR (50 μM) were measured under analogous conditions, and served as the basis for the determination of the amount of Zn^{2+} released from LF.

2.6. Intrinsic tryptophan fluorescence spectroscopy and data analysis

In a total volume of 0.8 mL, LF at a final concentration of 0.5 μM in Hepes buffer (50 mM, pH 7.4) was incubated at room temperature in the absence and presence of GdnHCl or urea (0–6 M) for 1 h or 24 h prior to monitoring protein fluorescence. Samples of apoLF were prepared and measured analogously except for the inclusion of dipicolinic acid at a final concentration of 0.1 mM to prevent partial reconstitution by trace amounts of Zn^{2+} present in the medium. Samples of Zn^{2+}-reconstituted apoLF were prepared by exposing the apoprotein (2 μM) to Zn^{2+} (5 μM) for 1 h prior to diluting the sample with Hepes buffer containing the desired amount of denaturant to achieve final concentrations of 0.5 μM and 1.25 μM with respect to the protein and metal ion, respectively. It is important to note that Zn^{2+}-reconstituted apoLF obtained in this manner was found to regain its full catalytic activity.

Steady-state fluorescence emission spectra of ZnLF, apoLF and Zn^{2+}-reconstituted apoLF were recorded at 20 °C using an OLIS RSM1000 spectrofluorometer (Bogart, GA) equipped with a 150 W Xenon arc lamp, a photon counter and a Julabo CF31 water bath (Allentown, PA) for temperature control. The excitation wavelength was set to 295 nm with a 5 nm bandpass. Emission spectra were obtained from 300 to 400 nm in 0.5 nm increments using an integration time of 1 s, and a bandpass of 4 nm. All spectra were processed by subtraction of spectra obtained for the corresponding denaturant/buffer samples in the absence of protein, followed by smoothing using the OLIS GlobalWorks software.

The degree of LF unfolding was assessed on the basis of the fluorescence intensities at 333 nm (FI_{333}), the wavelength at which

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