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High metal substitution tolerance of anthrax lethal factor and characterization of its active copper-substituted analogue

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ABSTRACT

Anthrax lethal factor (LF) is a zinc-dependent metalloendopeptidase and a member of the gluzincin family. The current report demonstrates a high metal substitution tolerance of LF atypical of gluzincins and other zinc-dependent metalloproteases. Mn^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+} and Cd^{2+} were found to reactivate the apoprotein of LF to a level either comparable to or significantly higher than that noted for the native zinc enzyme. The most active form of LF was obtained with Cu^{2+}, a surprising observation since most Cu^{2+}-substituted zinc proteases display very low activity. Cu^{2+}-substituted LF (CuLF), prepared by direct exchange and by apoprotein reconstitution methodologies, displayed a several-fold higher catalytic competence towards chromogenic and fluorogenic LF substrates than native LF. CuLF bound Cu^{2+} tightly with a dissociation constant in the femtomolar range. The electron paramagnetic resonance spectrum of CuLF revealed the protein-bound metal ion to be coordinated to two nitrogen donor atoms, suggesting that Cu^{2+} binds to both active site histidine residues. While ZnLF and CuLF (prepared by direct exchange) were capable of killing RAW 264.7 murine macrophage-like cells, apoLF and all metal-reconstituted apoprotein preparations failed to elicit a cytotoxic response. Competition experiments using apoLF/ZnLF mixtures demonstrated the propensity of apoLF to relieve ZnLF-induced cell death, suggesting that both protein forms can compete with each other for binding to protective antigen. The lack of cytotoxicity of apoLF and its metal-reconstituted variants likely originates from structural perturbations in these proteins which might prevent their translocation into the cytoplasm.

1. Introduction

Anthrax is an infectious bacterial disease caused by *Bacillus anthracis*, and has been the topic of considerable interest as an animal and human pathogen, as well as a potential component of biological weapons [1]. Three proteins, protective antigen (PA), edema factor (EF), and lethal factor (LF), collectively known as the anthrax toxin, play a pivotal role in the manifestation of the disease [2]. PA (in its heptameric or octameric form) is a pore-forming protein which mediates the entry of LF and EF from the endosome into the host cell cytosol [3–7]. EF is a calcium and calmodulin-dependent adenylyl cyclase responsible for the generation of supraphysiological amounts of cyclic AMP, thus resulting in a disruption of water homeostasis [8,9]. LF is a zinc-dependent metalloendopeptidase responsible for the cleavage of most members of the mitogen-activated protein kinase kinase (MAPKK) family of signalling proteins near their N-termini [10–13]. Furthermore, LF has recently been shown to facilitate inflammasome activation and macrophage death by removing an N-terminal segment from NOD-like receptor protein 1 (Nlrp1) [14–17].

The zinc ion in LF is coordinated to the side chains of His686, His690, and Glu735, and to a water molecule which serves as the nucleophile in the peptide bond hydrolysis reaction [13,18]. In addition, Glu687, which is part of the thermolysin-like HExxH consensus motif [19], has been proposed to serve as a general base in the catalytic mechanism of LF by activating and orienting the zinc-bound water molecule for proper nucleophilic attack on the carbonyl carbon atom of the scissile peptide bond [13]. In view of the aforementioned amino acid residues involved in zinc coordination and water activation, LF can be classified as a gluzincin [19].

Initial investigations on the metal requirement of LF using reconstitution assays involving apoLF have revealed the protein's stringent requirement for Zn^{2+} for catalytic function. Indeed, apoLF preparations supplemented with other transition metal ions such as Mn^{2+}, Co^{2+}, Ni^{2+} and Cu^{2+} were found not to regain a significant degree of catalytic competence (typically below 2% of the activity of native ZnLF) [20], although some of these ions were found to effectively compete with Zn^{2+} for protein binding in earlier radiolabeling studies [21]. Furthermore, Ca^{2+} and Mg^{2+} have previously been shown to be required to restore (some of) the activity of apoLF by Zn^{2+} and other divalent transition metal ions [20,22]. However, recent studies have revealed the requirement for these alkaline earth metals not to be an inherent feature

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of LF, but instead to be dependent on the protocol of apoLF preparation. For instance, while apoLF generated by dialysis following exposure of ZnLF to chelators exhibited a dependence on Ca^{2+} and Mg^{2+} for enzyme activation by Zn^{2+} or Co^{2+}, the apoprotein recovered by centrifugal filtration (subsequent to chelator treatment) was found not to rely on alkaline earth metals for full reconstitution of LF's catalytic activity [23]. The dichotomy of these results has been attributed to the presence of residual chelator in dialyzed (but not centrifugally-filtered) apoLF samples, which can lead to complications in the interpretation of reconstitution data [23]. Although Zn^{2+} and Co^{2+} have been found to reconstitute apoLF in the absence of any other supplementary metal ions, the ability of other transition metal ions to restore the catalytic competence of the apoprotein has not been (re)investigated.

The current report describes a reinvestigation of the propensity of apoLF to regain its catalytic function upon supplementation with various transition metal ions. Our results demonstrate that, with the exception of Fe^{2+}, all biologically relevant 3d metals are capable of restoring the apoprotein's function. Furthermore, reconstitution of apoLF by Cu^{2+} was found to lead to an active enzyme which significantly exceeds the catalytic competence of ZnLF, an unusual phenomenon given that the vast majority of Cu^{2+}-substituted zinc proteases display markedly reduced activities compared to those recorded for their native zinc counterparts [24]. Consequently, Cu^{2+}-substituted LF was prepared by two independent methods (one involving the direct exchange of ZnLF's Zn^{2+} ion by Cu^{2+}, the other based on the reconstitution of apoLF with Cu^{2+}), and characterized kinetically and spectroscopically. In addition, the effect of PA supplemented with apo- and metal-substituted LF on macrophage cell viability was assessed revealing all apoLF preparations (be they metal-deficient or metal-exposed) to be non-cytotoxic.

2. Experimental

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 Corporation (Cambridge, ON, Canada). MAPKKide was purchased from List Biological Laboratories (Campbell, CA). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO). All metal salts (chlorides of Mn^{2+}, Cd^{2+} and Co^{2+}; sulfates of Zn^{2+}, Fe^{2+}, Ni^{2+} and Cu^{2+}) were of analytical reagent grade, and contained ≤0.001% Zn^{2+}. All solutions were prepared using MilliQ ultrapure water (≥18.2 MΩ cm resistivity), and buffers were depleted of trace metals by treatment with Chelex-100 resin.

2.2. Preparation of ZnLF, apoLF and CuLF

Wild-type LF (ZnLF) was isolated and purified as outlined previously [25], and contained 1.0 (±0.1) Zn^{2+}/protein molecule. ApoLF was prepared as described earlier with some minor modifications [23]. In brief, ZnLF (10 μM) in Hepes buffer (50 mM, pH 7.4) was exposed to 10 mM ethylenediaminetetraacetic acid (EDTA) and 1 mM dipicolinic acid (DPA) for 48 h at 4 °C prior to removal of excess chelator and concentration of the protein using an Amicon Ultra-15 centrifugal filter (30 kDa MWCO; Millipore, Bedford, MA). The absence of protein-bound Zn^{2+} and residual chelator (which can interfere with the proper interpretation of metal reconstitution data [26]) following centrifugal filtration was ascertained with 4-(2-pyridylazo)resorcinol (PAR) according to published protocols [23]. ApoLF obtained in this manner contained ≤0.03 Zn^{2+}/LF molecule. CuLF was prepared by two independent methods, one involving the direct exchange of Zn^{2+} in ZnLF with Cu^{2+}, the other relying on the reconstitution of apoLF with Cu^{2+}. Regarding the first method, 2 mL of purified ZnLF (20 μM) was loaded onto a Q-Sepharose column (5 mL bed volume) equilibrated with Hepes buffer (50 mM, pH 7.0). Following immobilization of the protein, its Zn^{2+} ion was allowed to exchange with Cu^{2+} by passing 25 mL of $CuSO_4$ (0.1 mM) in Hepes buffer through the column using a flow rate of 1 mL/min. Excess Cu^{2+} and released Zn^{2+} were removed by washing the column with 50 mL of Hepes buffer containing 100 mM NaCl. The metal-exchanged protein was eluted from the column using 20 mL of Hepes buffer containing 350 mM NaCl prior to being concentrated (to 100–180 μM) by Amicon Ultra-15 filtration. In addition to the preparation of CuLF by direct exchange ($CuLF^{de}$), the protein was obtained by reconstitution of apoLF with Cu^{2+} ($CuLF^{rec}$). In a typical procedure, 125 μL of apoLF (150 μM) in Hepes buffer (50 mM, pH 7.4) was reconstituted with 0.96 mol-equivalents of Cu^{2+} in a step-wise fashion by slowly supplementing the protein with 5 × 5 μL of an aqueous Cu^{2+} stock solution (720 μM) at room temperature. The step-wise addition of small aliquots of the metal ion solution to apoLF was necessary to avoid the precipitation of $Cu(OH)_2$ in Hepes-buffered solutions at neutral pH [27]. The homogeneity of all LF preparations was assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis according to the method of Laemmli [28]. The concentration of LF was determined spectrophotometrically at 280 nm using an extinction coefficient of 74,200 M^{-1} cm^{-1} [23].

2.3. Enzyme assays

The enzymatic activity of LF was determined spectrophotometrically (at 405 nm) in Hepes buffer (50 mM, pH 7.4) at room temperature as described in the literature, using *S*-*p*NA (10 μM) as the chromophoric substrate [23,29]. In a few instances, the catalytic competence of the enzyme was assessed also with the aid of the fluorogenic MAPKKide substrate [30]. MAPKKide is a peptide derived from the MAPKK-2 substrate of LF harbouring an N-terminally linked *o*-aminobenzoic acid moiety (donor fluorophore) 2,4-dinitrophenyl group, C-terminal to the cleavage site, serving as the acceptor chromophore. In a typical assay, ZnLF (50 nM) or $CuLF^{de}$ (10 nM) in Hepes buffer (50 mM, pH 7.4) was incubated for 1 min at room temperature prior to the addition of MAPKKide (5 μM final concentration), and the increase in fluorescence intensity at 420 nm (following excitation at 320 nm), a consequence of the decrease in intramolecular quenching, was recorded using an OLIS RSM-1000 spectrofluorometer (Bogart, GA). Steady-state kinetic parameters (K_M, k_{cat}) for the hydrolysis of *S*-*p*NA by $CuLF^{de}$ and $CuLF^{rec}$ (both 10 nM) were estimated by (non-linear least squares) fitting of the initial velocity data to the Michaelis–Menten equation using Grafit 4.0 (Erithacus Software Ltd., Staines, UK), and a $\Delta\varepsilon_{405\ nm}$ value of 9920 M^{-1} cm^{-1} [29].

2.4. Determination of metal content

The Zn^{2+} content of ZnLF and apoLF was determined with the aid of PAR according to published protocols [23]. The metal content (both Cu^{2+} and Zn^{2+}) of $CuLF^{de}$ was assessed using the Zincon/EDTA method as described in the literature with minor modifications [31]. In brief, $CuLF^{de}$ at a final concentration of 10 μM was introduced into borate buffer (50 mM, pH 9.0) containing urea (6 M) and Zincon (40 μM). Following incubation of the mixture for 5 min at room temperature, the absorbance at 615 nm (i.e., at the isosbestic wavelength, at which the extinction coefficients of the Zn^{2+}:Zincon and Cu^{2+}:Zincon complexes are identical) was determined. The total concentration of metal (Zn^{2+} and Cu^{2+}) in the sample was calculated on the basis of the linear relationship between the absorbance at 615 nm and the concentration of metal in a set of Zn^{2+} (or Cu^{2+}) standards (0–20 μM) prepared and measured under analogous conditions. The concentration of Cu^{2+} was subsequently assessed by adding 1 mM EDTA (which is capable of rapidly demetallating Zn^{2+}:Zincon, but not the Cu^{2+}:Zincon complex [31]) to the protein sample, followed by the measurement of the absorbance at 615 nm. The concentration of Zn^{2+} in the sample was calculated by subtracting the obtained concentration of Cu^{2+} from the concentration of total metal (i.e., Cu^{2+} and Zn^{2+}).