Journal of Inorganic Biochemistry 182 (2018) 1–8

Contents lists available at ScienceDirect

Journal of Inorganic Biochemistry

journal homepage: www.elsevier.com/locate/jinorgbio

An investigation of the pH dependence of copper-substituted anthrax lethal factor and its mechanistic implications

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ARTICLE INFO

Keywords:
Zinc
Copper
Lethal factor
Proteases
Metal substitution
Enzyme mechanism

ABSTRACT

Anthrax lethal factor (LF) is a zinc-dependent endopeptidase involved in the cleavage of proteins critical to the maintenance of host signaling pathways during anthrax infections. Although zinc is typically regarded as the native metal ion in vivo, LF is highly tolerant to metal substitution, with its replacement by copper yielding an enzyme (CuLF) 4.5-fold more active than the native zinc protein (at pH 7). The current study demonstrates that by careful choice of the buffer, ionic strength, pH and substrate, the activity ratio of CuLF and native LF can be increased to > 40-fold. Using a fluorogenic LF substrate, such optimized assay conditions can be exploited to detect LF concentrations as low as 2 pM. In contrast to the zinc form, CuLF was found to be inhibited by bromide and iodide ions, to be resistant to metal loss under acidic conditions, and to display a sharp pH dependence with significantly shifted alkaline limb towards more acidic conditions. The alkaline limb in the enzyme's pH profile is suggested to originate from changes in the protonation state of the metal-bound water molecule which serves as the nucleophile in the catalytic mechanism. Based on these observations and studies on other zinc proteases, a minimal mechanism for LF is proposed.

1. Introduction

Anthrax lethal factor (LF) is a zinc-dependent endopeptidase, and, next to protective antigen (PA) and edema factor (EF), one of the three protein components of the anthrax toxin secreted by *Bacillus anthracis*, the etiological agent of anthrax [1,2]. To reach its cellular target in the host cell cytoplasm, LF requires translocation through the endosomal membrane in a pH-dependent process mediated by the PA pore [3–6]. Once inside the cytoplasm, LF exerts its function by removing the N-terminal segment of most mitogen-activated protein kinase kinases (MAPKKs), hence leading to an impairment of critical intracellular signaling pathways [7–9]. In addition, LF has been identified more recently as a facilitator of inflammasome activation in rodents by being capable of cleaving NOD-like receptor protein 1 (Nlrp1) near its N-terminus [10–13].

The zinc ion in LF has been shown to be coordinated to the side chains of three amino acid residues (His686, His690 and Glu735) as well as to a water molecule, which acts as the nucleophile attacking the carbonyl carbon atom of the scissile peptide bond during substrate hydrolysis [9]. In addition to the coordinating residues, Glu687 and Tyr728 have been implicated in fulfilling critical roles in the enzyme mechanism by polarizing the nucleophilic water molecule and stabilizing the amine leaving group following peptide bond cleavage, respectively [9,14]. In view of the participation of these amino acid residues in metal ion binding and catalysis, LF can be classified as a gluzincin, harbouring the prototypical thermolysin-like HExxH…E consensus motif [15].

Although the Zn^{2+} ion appears to be the most likely occupant of LF's active site in vivo, recent studies have shown that the enzyme possesses both the propensity to readily exchange its active site metal ion [16] and an unusually high metal substitution tolerance [17]. For instance, LF has been found to be catalytically functional with Mn^{2+}, Cd^{2+}, Co^{2+} and Ni^{2+}, with the latter two ions rendering the enzyme twice as active as the native protein (ZnLF). Furthermore, the activity of Cu^{2+}-substituted LF (CuLF) was shown to exceed that of ZnLF by a factor of four to five. Although a twofold activation by Co^{2+} is not surprising in view of LF's close relative thermolysin (TL) being twice as active with this metal ion [18], the high level of activity displayed by CuLF is quite unusual since most zinc proteases (including TL) possess little activity when substituted with Cu^{2+} [17–19]. In addition, kinetic analysis of the Zn^{2+}-to-Cu^{2+} exchange process in LF has revealed the reaction to be rapid (half-time of exchange ~10 s), and to proceed via an associative-type mechanism [16]. Finally, the combination of CuLF and PA was found to be cytotoxic to murine macrophage-like RAW 264.7 cells, suggesting that the enzyme might be capable of cleaving MAPKKs in vivo [17].

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https://doi.org/10.1016/j.jinorgbio.2018.01.015
Received 11 October 2017; Received in revised form 18 December 2017; Accepted 22 January 2018

Since the molecular basis underlying the high activity of CuLF remains to be established, the current study sought to more closely examine the catalytic properties of CuLF (using ZnLF as a reference) by investigating the effects of buffer concentration, ionic strength, pH and the choice of substrate on enzyme function. We here demonstrate that an optimization of CuLF activity can be achieved using slightly acidic conditions (pH 6.5), and a total ionic strength of 125 mM. Under such conditions, CuLF/ZnLF activity ratios can reach unprecedented values (as high as 41). Furthermore, the pH profile of CuLF was found to be very narrow and distinct from that reported previously for ZnLF, especially in the slightly alkaline pH region. Based on these observations and data accumulated on a variety of zinc proteases, a mechanism for LF-mediated peptide bond hydrolysis is advanced, which involves a pentacoordinate metal ion and a metal-bound water molecule serving as the nucleophile.

2. Experimental

2.1. General

Custom-synthesized chromogenic anthrax lethal factor substrate II, *S*-*p*NA (Ac-Gly-Tyr-β-Ala-Arg-Arg-Arg-Arg-Arg-Arg-Arg-Arg-Val-Leu-Arg-*p*NA; *p*NA = *para*-nitroanilide) was obtained from Biomatik Corp. (Cambridge, ON, Canada). Anthrax lethal factor substrate III (*S*-AMC; Ac-Gly-Tyr-β-Ala-Arg-Arg-Arg-Arg-Arg-Arg-Arg-Arg-Val-Leu-Arg-AMC; AMC = 7-amido-4-methylcoumarin) was purchased from Millipore (Bedford, MA). MAPKKide was obtained from List Biological Laboratories (Campbell, CA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Unless stated otherwise, all solutions were prepared with MilliQ ultrapure water (≥18.2 MΩ cm resistivity). To minimize contamination by trace metals, buffers were treated with Chelex-100 resin (Sigma-Aldrich).

2.2. Isolation and purification of LF

Zinc-containing anthrax lethal factor (ZnLF) was isolated from *Bacillus megaterium* harbouring the pWH1520-LF plasmid (MoBiTec, Göttingen, Germany), and purified as described previously [20]. The demetallated apoform of LF (apoLF) was prepared by treating ZnLF with ethylenediaminetetraacetic acid (EDTA) and dipicolinic acid (DPA) according to published protocols [17]. The Zn^{2+} content of all ZnLF and apoLF preparations was determined with the aid of the chromophoric chelator 4-(2-pyridylazo)resorcinol as outlined previously [21], and was found to be 1.0 (± 0.1) Zn^{2+} per LF molecule for the native enzyme, and < 0.05 Zn^{2+} for the apoprotein. Reconstitution of apoLF to CuLF was achieved by exposure of the apoprotein (1 μM) in HEPES buffer (50 mM, pH 7.4) to 5 μM $CuSO_4$ for 30 min at room temperature. It is important to note that an increase in the concentration of Cu^{2+} (beyond 5 μM) did not lead to a further activation of the enzyme, a feature indicative of full reconstitution. Protein concentrations were determined spectrophotometrically at 280 nm using an extinction coefficient of ε = 74,200 M^{-1} cm^{-1} [21].

2.3. Enzyme assays

The enzymatic activity of LF was typically assessed as described in the literature [17,21]. Unless indicated otherwise, ZnLF and CuLF in HEPES buffer (50 mM, pH 7.4) at final concentrations of 50 nM and 10 nM, respectively, were supplemented with 10 μM *S*-*p*NA (using a 200 μM stock solution in HEPES buffer) prior to recording the progress of substrate hydrolysis at 405 nm (for 60 s at 20 °C) with the aid of a Cary 60 UV–Vis spectrophotometer (Agilent Technologies, Mississauga, ON). In studies aimed at maximizing the activity of CuLF, the dependence of the enzyme's catalytic competence on the concentration of MES buffer, the ionic strength, and the pH value was assessed. The dependence of CuLF activity on the concentration of MES was monitored using 10 μM *S*-*p*NA (as described above) following incubation of the protein in the buffer (5–100 mM) at pH 6.5. It is important to note that the pH value remained constant at pH 6.5 over the duration of the enzyme assay only when MES concentrations ≥5 mM were used. For each assay, the total ionic strength of the buffer (*I*) was adjusted to 125 mM by the addition of the appropriate amount of NaCl. In the case of the 100 mM MES buffer at pH 6.5, for instance, the concentration of the charged, conjugate base form of MES (which contributes to the ionic strength) is 69 mM based on the buffer's pK_a value of 6.15 (at 20 °C, [22,23]). Hence, the adjustment of *I* to 125 mM required the addition of NaCl at a final concentration of 56 mM.

The assessment of CuLF activity as a function of ionic strength was achieved similarly by incubating the enzyme in MES (25 mM, pH 6.5) in the absence and presence of NaCl prior to the initiation of the assay by the addition of substrate. In a few instances, NaCl was replaced with NaBr and NaI to investigate with influence of the anion on CuLF activity. The effect of pH on CuLF activity was investigated by incubating the protein for 5 min in NaCl-supplemented MES buffer (25 mM; *I* = 125 mM) at the desired pH value (adjusted using 1 M NaOH) prior to the addition of *S*-*p*NA (10 μM).

The buffer and pH dependence of the activity of CuLF (and ZnLF serving as a reference) was investigated also using the two fluorogenic LF substrates *S*-AMC and MAPKKide. While *S*-AMC is a fluorescent analogue of *S*-*p*NA harbouring the same 14-mer peptidic backbone but a C-terminal 7-amido-4-methylcoumarin group instead of the *p*-nitroanilide moiety [24], MAPKKide is a FRET peptide designed from the MAPKK-2 substrate of LF containing an N-terminal *o*-aminobenzoic acid group as the donor fluorophore, and a C-terminal 2,4-dinitrophenyl moiety as the acceptor chromophore [25,26]. All assays were performed at 20 °C using ZnLF and CuLF (at final concentrations of 50 nM and 10 nM, respectively) in the desired buffer (HEPES (50 mM, pH 7.4), MOPS (50 mM, pH 7.4) or MES (25 mM, pH 6.5 ± NaCl)). Following the addition of substrate (5 μM), the progress of the hydrolysis reaction was monitored for 60 s (in 0.5 s intervals) using an OLIS RSM-1000 spectrofluorometer (Bogart, GA) equipped with a 150 W Xe arc lamp and a Julabo CF31 water bath (Allentown, PA) for temperature control. The excitation/emission wavelengths were set to 360 nm/460 nm and 320 nm/420 nm to record the hydrolysis of *S*-AMC and MAPKKide, respectively. For all measurements, the monochromator bandpasses were set to 5 nm for excitation and 4 nm for emission.

The estimation of the limit of detection of CuLF was achieved by recording the progress of *S*-AMC hydrolysis in MES (5 mM, pH 6.5, *I* = 125 mM) supplemented with 0.1% (w/v) bovine serum albumin at 20 °C for 15 min with a final enzyme concentration of 10 pM. The standard deviation of four such (independent) measurements was multiplied by the appropriate Student's t value (three degrees of freedom) to obtain the method detection limit (95% confidence interval).

2.4. Determination of specific activities and steady-state kinetic parameters

Specific activities (expressed in μmoles of substrate hydrolyzed per minute per mg of protein) were determined from the slope of the trendline through the initial linear portion of the hydrolysis progress curves. While an extinction coefficient ($\Delta\varepsilon_{405\ nm}$) of 9920 M^{-1} cm^{-1} [24] was employed to estimate LF's specific activity towards the hydrolysis of *S*-*p*NA, those towards the cleavage of *S*-AMC and MAPKKide were assessed based on the fluorescence intensity of 5 μM fully hydrolyzed fluorogenic substrate (monitored at 460 nm and 420 nm, respectively) serving as a reference. Steady-state kinetic parameters (K_M, k_{cat}) for the hydrolysis of *S*-*p*NA and *S*-AMC (using a substrate concentration range of 1 μM to 15 μM) were determined 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).