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Implication of pH in the catalytic properties of *anthrax* lethal factor

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Abstract

The anthrax lethal factor (LF) is a Zn^{2+}-endopeptidase specific for mitogen-activated protein kinase kinases (MAPKKs), which are cleaved within their N-terminal region. Much line of effort was carried out to elucidate the catalytic activity of LF for designing the inhibitor and to understand the cellular mechanism of its cytotoxicity. Current assay methods to analyze the LF activity have been based on a synthetic peptide, consisting of 15–20 residues around being cleaved. However, there are accumulating reports that the region distal to cleavage site is required for the LF-mediated proteolysis of substrate. In this study, we demonstrate the catalytic properties of LF, using the full-length native substrate, MEK. We described the catalytic properties of LF focused on the effects of the pH alteration, which was encountered during the endocytosis of lethal toxin, and of the requirement for metal ions. We present the first evidence that additional metal ions are required for the LF catalyzed hydrolysis of native substrate, and that the pH alteration causes a significant change of catalytic properties of LF.

Keywords: Lethal factor; Catalytic properties; pH alteration; Metal ion dependence

Anthrax is a zoonotic disease whose etiologic agent is a gram-positive sporulating bacterium, *Bacillus anthracis* ([1–3] for review). Anthrax toxins are comprised of protective antigen (PA; 83 kDa), lethal factor (LF; 90 kDa), and edema factor (EF; 89 kDa), of which no single component is toxic but the combination of PA with either LF (lethal toxin, LeTx) or EF (edema toxin, EdTx) leads to pathogenesis in laboratory animals. Anthrax toxin, like other bacterial toxins [4], fits the A–B model of classification of toxins, where B (PA in this case) is the binding moiety, which binds to the cell surface receptors, and LF/EF are alternate catalytic A moieties.

LeTx as the name suggests is lethal for several species. Mouse peritoneal macrophages and macrophage-like cell lines, such as J774A.1, and RAW 264.7, etc., are sensitive to this toxin [5–7]. In addition, LF has been known to exhibit cytotoxicity toward animals, an activity likely to be important for evasion of host defense. Recently, much effort has been concentrated on demonstration of the cellular mechanism underlying LF-mediated cytotoxicity. Pellizzari et al. [8] showed that low levels of LeTx cleave MAPKK-3, inhibiting release, but not production, of the proinflammatory mediator, NO, and tumor necrosis factor-α (TNF-α). In contrast, high levels of the toxin lead to lysis of macrophage within a few hours. Furthermore, it has been reported that the dismantling of the p38 MAPK module represents a strategy used by *B. anthracis* to paralyze host innate immunity [9]. However, the exact mechanism of cell death and the cellular target of lethal factor remain to be further elucidated.

The identification of a physiological target of LF is important to design specific inhibitors and to expand the knowledge of pathogenesis of LeTx. It has been extensively shown that LF displays metalloproteolytic activity directed toward the N-terminus of mitogen-activated protein kinase kinases (MAPKKs) [10–13]. Because N-terminal extension is required for interactions with both MAPKs and MKK kinases (MKKKs), this cleavage prevents MAPK activation. Biochemical approaches have led to the determination of the conserved cleavage site of substrate. Hydrophobic amino acids seem to be

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preferred in positions P2′ and P1′, and basic residues are located at N-terminus of the cleavage site [14–16]. This result was complemented by the structural model study for the active site of LF. X-ray crystallographic image clearly shows the existence of a broad deep groove, 40 Å long, which is contiguous with the active site [17]. This groove has, in general, a charged potential, containing clusters of glutamic acid/aspartic acid, as well as glutamine/asparagine residues. A strongly acidic region of its base rationalizes the preference for basic residues in the MAPKK substrates located 3–6 residues upstream of the cleavage site. Although LF displays a wide spectrum of proteolytic activity on the MAPKK family, except for MEK5, this active pocket is not closed at N-terminal end of all these members. Therefore, other surfaces may provide additional docking sites for other domains on the MAPKK, because fragments of MAPKK-1 lacking the N-terminal tail also bind to LF in a yeast two-hybrid screen [12]. Furthermore, Chopra et al. [18] showed that C-terminal regions, which were distal to the cleavage site, were essential for LF-mediated proteolysis of its substrate, MEK.

In this study, the proteolytic properties of LF have been investigated using native substrate, GST-MEK1, which allowed us to more extensively study the relationship between enzyme and substrate in terms of binding/catalysis. We present the first evidence that an additional metal ion is required for the LF catalyzed hydrolysis of native substrate and that the pH alteration, which mimics endocytosis, causes a significant change of catalytic properties of LF. These findings provide new insights into the design of novel inhibitors of LF and into the understanding of the macrophage toxicity of LF.

Materials and methods

Materials. The *Escherichia coli* strain, BL21(*DE3*), was used as an expression host strain. Cell culture media (Bacto-tryptone, Bacto-yeast extract) were purchased from Difco. Restriction enzyme, *Pfu* DNA polymerase, T4 DNA ligase, and other cloning reagents were from Promega. Other chemicals, including 1,10-phenanthroline, isopropyl-1-thiogalactopyranoside (IPTG), and buffer chemicals, were from Sigma.

Construction and purification of recombinant lethal factor. Construction of recombinant lethal factor (GST-LF) and its purification were carried out as previously reported [19,20]. Briefly, the gene coding to lethal factor was isolated from *Bacillus anthracis* sterne and amplified by the polymerase chain reaction, followed by subcloning into the bacterial expression vector, pGEX-KG. The plasmid containing LF was introduced into the expression host, BL21(DE3), which has relatively low protease activity. Protein expression was induced by adding IPTG to the final concentration of 1 mM and cells were harvested at 5 h postinduction. Cell pellets were resuspended in PBST buffer (150 mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4, pH 7.4, 2 mM EDTA, 1% Triton X-100, and 0.1% BME) and incubated on ice for 10 min, followed by lysis with ultrasonication. Resultant lysates were directly incubated with glutathione (GSH)–Sepharose 6B resin, which was pre-equilibrated with PBST buffer, at 4 °C for 30 min with mild shaking. After incubation, target protein bound to the resin was separated from the total cell extracts by short centrifugation and vigorously washed with PBST buffer containing 5 mM GSH and 250 mM NaCl several times. Recombinant LF was eluted by a step gradient with 20 mM GSH (buffered with 50 mM Tris, pH 8.0) at 4 °C and dialyzed against the buffer containing 20 mM Tris, pH 8.0, 2 mM EDTA, and 10% glycerol. Purified protein showed high homogeneity onto 10% SDS–PAGE analysis. The concentration of protein was determined by Bradford reagent according to manufacturer's direction.

Proteolytic assay of lethal factor. Enzyme activity was determined by the cleavage of recombinant substrate, GST-MEK, which was expressed as a fusion protein at the N-terminus of MEK. Proteolytic activity of LF was performed essentially as previously described [19] with the following modification. Enzyme was preincubated with or without 10 mM of 1,10-phenanthroline at 37 °C for 20 min and then added to the reaction mixture containing 25 mM phosphate buffer, pH 7.4, 20 mM NaCl, 1 mM DTT, 0.1 mM $ZnCl_2$, 0.1 mM $CaCl_2$, 10 mM $MgCl_2$, 10% glycerol, and 1 μM substrate (GST-MEK). The reaction was carried out at 37 °C for 60 min and terminated by addition of 5× SDS-loading buffer, followed by boiling. Reaction products were resolved on 10% SDS–PAGE. To determine the metal effects on proteolytic activity, apo-enzymes were prepared by dialysis against 10 mM EDTA and 1 mM of 1,10 phenanthroline after the elution step of purification and then proteins were further dialyzed against a buffer containing 20 mM Tris, pH 7.4, at 4 °C for 16 h with four times buffer changes.

Alteration of pH. First, enzymes were incubated in the appropriate buffer at room temperature for 30 min and then challenged to pH alteration by the addition of 10 volume excess of indicated pH buffer for additional 30 min. After incubation, the mixture containing enzyme was concentrated to 1/10 volume by ultafiltration unit (Amicon Unltra-15, Molecular weight limit of 10,000, Millipore). pH was adjusted to a neutrality by adding 10 volume excess of 25 mM phosphate buffer, pH 7.4, and incubated at room temperature for 1 h. pH change was confirmed at every step.

Data analysis. Proteolytic activity of LF was estimated from the decrease substrate band intensity compared to the reference on 10% SDS–PAGE, using densitometer (Bio-Rad). LF-mediated cleavage of the substrate, GST-MEK, resulted in two fragments, a 44 kDa one (Ile9 to the end of MEK1) and a 28 kDa one (GST moiety containing N-terminus (Met1–Pro9) of MEK1). LF activity was calculated by the measurement of two band-intensity ratios between substrate GST-MEK (72 kDa) and one of its cleaved products, MEK (Ile9 to C-terminal end, 44 kDa). Activity was normalized with the enzyme amount measured by densitometer on 10% SDS–PAGE. All representative data were from at least three independent experiments.

Results

Proteolytic assay of LF using full-length substrate, GST-MEK

The efforts to demonstrate the catalytic properties of lethal factor as a protease have been performed by the method based on a synthetic peptide, which was composed of the conserved sequence of MAPKK family around being cleaved [14–16,21]. However, considering the high specificity of LF toward its cognate substrate (MEK), the catalysis and binding properties of LF might not be fully elucidated by current methods. The crystal structure of LF suggested that there are other key factors in recognizing its substrate besides the sequence at the cleavage site [17]. Also, it has been documented

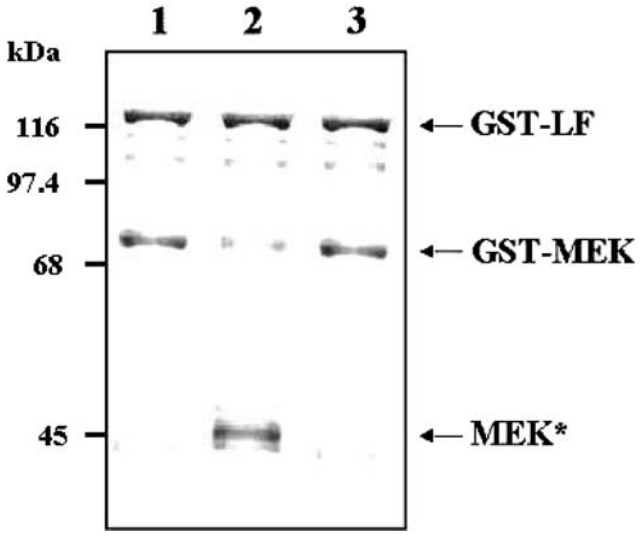

Fig. 1. The proteolytic activity assay using full-length GST-fusion substrate, GST-MEK. Activity of LF was determined as described in Materials and method. 0.5 μM of rLF was preincubated with (3) or without (1, 2) 10 mM of 1,10 phenanthroline, and the reaction was initiated by adding the reaction mixture containing full-length substrate (3.5 μM, GST-MEK). MEK* represents one of the cleaved products, MEK1 (Ile9-end). Lane 1, zero time point as a reference; lanes 2 and 3, reaction was carried out at 37 °C for 60 min.

that cosolvent additives including $MgSO_4$ might be necessary to preserve thermostability of toxin components [22]. Furthermore, it has been recently reported that the C-terminus of MAPKKs is necessary for substrate specificity and proteolytic activity of LF. The authors suggested that the LFIR (LF interacting region) in the C-terminal kinase domain of MEK1, adjacent to the proline-rich region, is essential for LF-mediated proteolysis of MEK1 [18]. Therefore, we have tried to demonstrate the properties of proteolytic activity of LF under the condition of native substrate, MEK, rather than short synthetic peptide. To do this, we prepared the substrate as a GST-fusion protein, GST-MEK, because the region being cleaved by LF is too close at N-terminus of MEK1 (Pro8–Ile9) to identify its cleavage. As shown in Fig. 1, recombinant LF cleaved the substrate MEK in their N-terminus and its cleavable activity was strongly inhibited by the addition of Zn^{2+} chelator, 1,10 phenanthroline. These results confirmed that LF is a Zn^{2+}-dependent endopeptidase as previously reported [14,15] and that our assay method using full-length substrate, GST-MEK, was sufficient to analyze the catalytic properties of LF.

Catalytic properties of LF about metal ions

It has been extensively documented that LF is a Zn^{2+}-dependent metalloprotease. The amino acid composition of residues 686–690 of LF is a well-known conserved Zn^{2+}-binding motif (HEXXH) and mutations in this region completely abolished enzyme activity [23]. In addition, near the center of the amino acid sequence (residues 315–416 of LF), there are five homologous repeats, which are proposed to form an EF-hand calcium-binding motif [14]. Furthermore, Ca^{2+} is reported to be required at several steps in toxification of cells by LeTx [24]. Indeed, in vitro assays using synthetic peptide fragment analysis by HPLC have shown that these two metal ions were essential and sufficient to proteolytic activity of LF. To examine the metal dependence of LF, we measured LF activity with the various combinations of divalent cations (Fig. 2). Apo-enzyme that was cleared of metal ions through dialysis with EDTA and phenanthroline showed no ability to cleave substrate. As individual metals are added back to LF, it is clear that Zn^{2+} ion is required, but this metal alone is not sufficient for full activity (about 40% of maximum activity, Fig. 2). Along with Zn^{2+}, Ca^{2+} and Mg^{2+} ions are also required for activity and the combination of Ca^{2+} and Mg^{2+} ions with Zn^{2+} ion showed maximum activity. These results suggest that each ion has a different binding site on the enzyme, and that an additional third metal ion, apart from Zn^{2+} and Ca^{2+} as previously reported [14,15], could be required in recognition of its cognate substrate (substrate specificity) or binding/catalysis on substrate cleavage. Other divalent cations, such as Ni^{2+} and Mn^{2+} showed similar results to that of magnesium ion (data not shown).

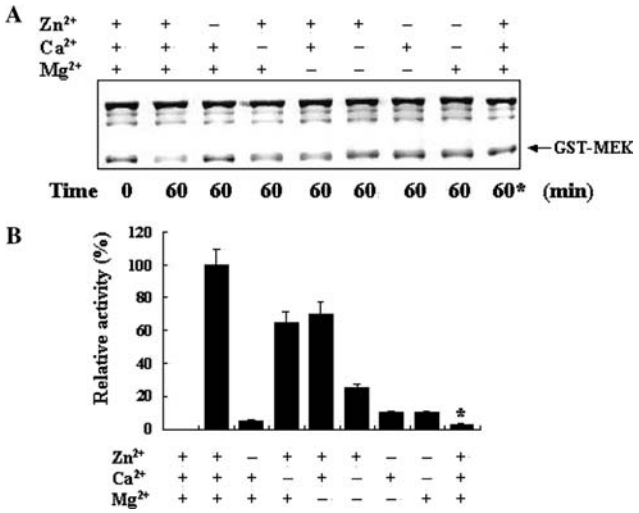

Fig. 2. Metal dependence on LF catalyzed substrate hydrolysis. (A) apo-enzymes were prepared by dialysis against buffer containing metal chelating agents, 10 mM EDTA and 1 mM of 1,10-phenanthroline. Proteolytic activity of LF was directly assessed in the reaction mixture containing the various combinations of indicated metal ions (0.1 mM $ZnCl_2$, 0.1 mM $CaCl_2$, or 10 mM $MgCl_2$). Representative data were one of the three independent experiments and showed similar results. (B) Enzyme activity was calculated from the decrease of substrate band intensity, using densitometry (Bio-Rad). Relative activity was calculated using a reaction with three metal ions as a standard (100%). The symbol (*) represents the reaction using the enzyme preincubated in 10 mM 1,10-phenanthroline at 37 °C for 20 min.

The effects of pH alteration on proteolytic activity

To display the cytotoxicity of LeTx, it must enter the target cells by endocytosis mediated by heptameric PA pore complex [2,25,26]. During this process, the PA–LF complex moves the acidic compartment and then releases the catalytic unit (LF) to cytosol [2,27]. In this respect, investigation of the effects of pH on LF activity seems to be important and pH alteration should be taken into consideration in the analysis of catalytic properties of LF. To address the effects of pH on LF activity, we first tried to determine the optimum pH for the enzyme activity in the range of pH 4.0–8.0. As shown in Fig. 3A, the results showed that the optimum pH of enzyme was near neutrality and significant activity was maintained at the pH range from 6.0 to 8.0. However, enzyme activity at pH 4.0 was nearly abolished. This result is in the line with the differential scanning calorimeter (DSC) data for the relationship between the thermal denaturation and the activity of LF at various pHs [28].

In addition, at the optimum pH 7.4, the kinetics of substrate cleavage were also determined. As shown in Fig. 3B, time kinetics was hyperbolic and the half-maximum was about 8 min. Until 15 min, the LF catalyzed cleavage of substrate was nearly linear under the condition of 0.5 μM LF and 3.24 μM GST-MEK.

Next, we planned to verify the effects of endocytosis on proteolytic activity of LF. Considering that there was little activity near the acidic pHs (Fig. 3A, at pH 4.0–5.0), it is of interest to investigate the catalytic properties of LF after pH change. To this aim, we performed LF activity assay using the enzymes incubated in serial buffer change system as described in Materials and methods. Recombinant LF was incubated in buffer at pH 4.0 for 30 min at room temperature and pH was restored to 7.4. The same operation was performed in the buffer at constant pH, 7.4, as a control to verify the effect of the process of buffer change. Interestingly, pH alteration induced a drastic change in the kinetic patterns for substrate. As shown in Fig. 4, time kinetics of

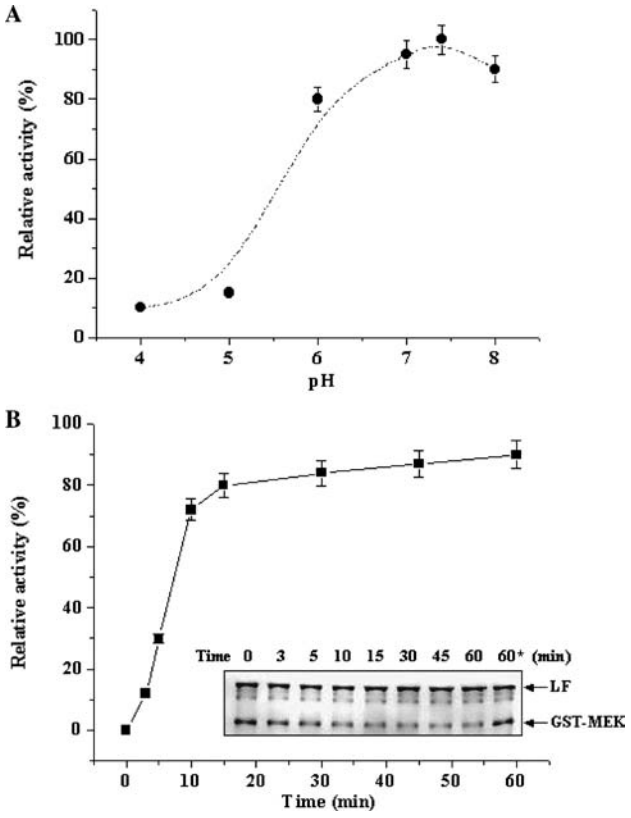

Fig. 3. The determination of catalytic properties of LF. (A) Optimum pH of LF. To determine the optimum pH of LF activity, the proteolytic activity of LF was measured in the pH range from 4.0 to 8.0 (pH 4.0–5.0, 25 mM acetate buffer, pH 5.0–7.0, 25 mM Mes buffer, pH 7.0–7.4, 25 mM phosphate buffer, and pH 7.4–8.0, 25 mM Hepes buffer). (B) Time kinetics on substrate. LF catalyzed substrate hydrolysis was monitored in a time-dependent manner. The proteolytic assay was performed in standard conditions containing all three metal ions, 0.1 mM Zn^{2+}, 0.1 mM Ca^{2+}, and 10 mM Mg^{2+}. At the indicated time point, aliquots were withdrawn and the reaction was terminated by adding 5× SDS–PAGE loading buffer, followed by boiling. The symbol (*) represents the reaction using the enzyme preincubated in 10 mM of 1,10-phenanthroline at 37 °C for 20 min.

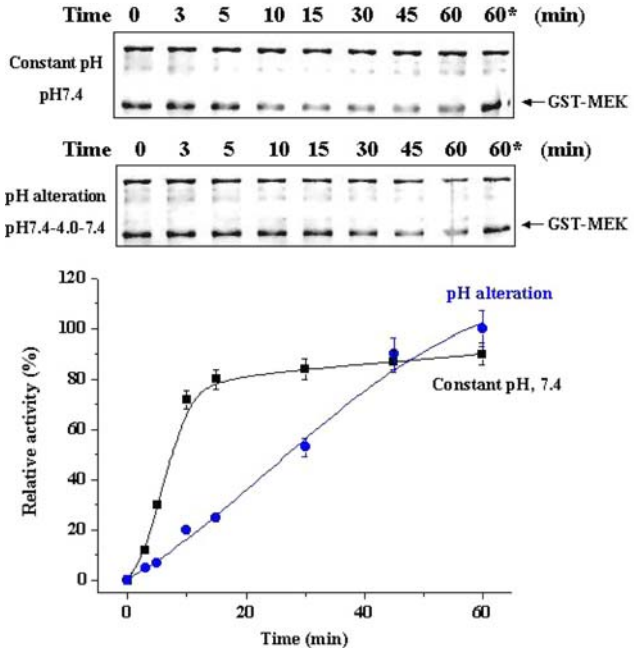

Fig. 4. The effect of pH alteration on LF activity. (A) To resolve the effect of pH change on LF activity, enzymes were incubated in the serial buffer system (pH 7.4 → 4.0 → 7.4, lower panel), which was changed to the indicated pH by the method described in Materials and methods. Enzyme assays were carried out according to the above procedures. Reactions were also performed in a constant pH buffer system (pH 7.4 → 7.4 → 7.4, upper panel) as an internal control. (B) Relative activity was estimated from the decrease of substrate band intensity, using densitometry (Bio-Rad) and each activity was compared to the reaction performed in constant pH for 60 min as a reference (100%). The symbol (*) represents the reaction using the enzyme preincubated in 10 mM of 1,10-phenanthroline at 37 °C for 20 min. Represented data were from at least three independent experiments.

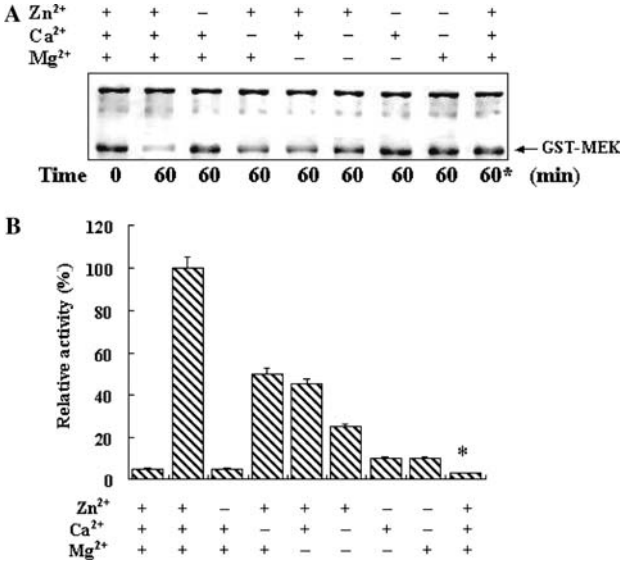

Fig. 5. Metal dependence on proteolytic activity of LF incubated in a serial buffer system (pH 7.4 → 4.0 → 7.4). Reactions were carried out essentially as described in Fig. 2. The symbol (*) represents the reaction using the enzyme preincubated in 10 mM of 1,10-phenanthroline at 37 °C for 20 min.

LF at the constant pH 7.4 (pH 7.4 → pH 7.0 → pH 7.4, upper panel) was similar to that of LF at the standard condition (Fig. 3B). However, time kinetics of LF incubated in consecutive pH changes (pH 7.4 → pH 4.0 → pH 7.4, lower panel) was significantly different to that of control. Proteolytic activity of LF at pH 4.0 was almost negligible, but was restored by the pH change. This result indicates that the structural transition of pH change is reversible, in contrast to the irreversible transition of thermal structural transition. Although the pH alteration induced a significant change in the time kinetics of enzyme on substrate, maximum activity of the enzyme was similar to that of the enzyme at the constant pH 7.4. Furthermore, pH alteration increased the dependence for a third metal ion, Mg^{2+}, on LF activity (Fig. 5). This result provides new insight into the relationship between LF and its substrate in vivo.

Taken together, our results suggest that the binding/catalysis of LF to native substrate requires additional metal ions, and that endocytosis induced subtle structural rearrangement of enzyme to have its characteristic function of LF in vivo.

Discussion

In this study, we determined the catalytic properties of LF using an assay method containing full-length substrate, GST-MEK, and provided the first evidence that the pH alteration, which was encountered in the process of endocytosis of *anthrax* toxin, induced a subtle change in the catalytic properties of LF.

Although our assay system was able to demonstrate the relationship between substrate and enzyme in terms of catalysis and binding, it is not easy to be considered for the sensitivity, rapidity, and quantification. Indeed, it has been reported that the efficacies of cleavage of the synthetic peptide substrates were determined, and one with a cleavable rate about 100-fold higher than the native MEK1 was documented [15,16]. However, the data that the C-terminal LFIR, the region distal to the cleaved site, are required for the LF-mediated proteolysis of MEK implicated that the characterization of catalytic properties of LF should be elucidated under conditions of native substrate rather than a short peptide containing only the cleaved sequences [18]. Crystal structures of the *anthrax* lethal factor explained the substrate specificity with its broad deep groove, which is 40 Å long and contiguous with the active site [17]. It consists of generally negative potential amino acids and displays restriction of access to the active site by potential substrates for the loops of a globular protein. In this structural proximity, synthetic peptide corresponding to N-terminal 16 residues of MEK2 seems to be properly fitted by basic side chains at the P1 position. However, this binding mode is relatively weak and the scissile bond is not within 5 Å of the catalytic core, Zn^{2+}-bound water. Furthermore, yeast two-hybrid experiments showed that MEK1 lacking the N-terminal tail also bound to LF, clearly indicating that additional docking sites were primary determinants to orientate and to bind enzyme to its native substrate [12]. It might be supported, at least in part, by our results that the native substrate MEK fused to a bulky GST moiety at its N-terminus was successfully cleaved by LF and that other metal ions other than Zn^{2+} and Ca^{2+} (essential ions for peptide cleavage) are required for maximum proteolysis against native substrate MEK. These results imply that regions other than the N-terminus being cleaved are important factors for the binding/catalysis of enzyme and that other metal ions could participate in this process. The high specificity toward substrate is also elucidated from studies for the other toxin zinc metalloprotease such as tetanus toxin (TeNT) and botulinum neurotoxins (BoNT) [29]. Among the group of zinc metallopeptidases, a particularity of these clostridial neurotoxins is high substrate selectivity, contrasting to the wide specificity of zinc proteases belonging to the thermolysin and metzincin families [30]. A mechanism that could account for the high degree of substrate specificity is the promotion of an active conformation of TeNT induced by the binding of the S1 (27–55) and S2 (82–93) domains of its substrate, synaptobrevin (regulator of neuroexocytosis) to exosites present on the toxin surface, as in the case of allosteric enzyme. This suggests that high selectivity might be due to a well-defined tertiary structure of its cognate substrate induced by residues far from cleavage site. In the case of LF, the same explanation is likely to be responsible for substrate specificity.

Moreover, we have showed that pH change causes a subtle alteration in catalytic properties of LF, a significant change of the time-kinetic curve (hyperbolic to sigmoid, Fig. 4B) and increased dependency on a third metal ion (Mg^{2+}) for maximum activity. These observations give important clues that endocytosis induces a drastic structural change to display its own catalytic properties and substrate specificity in vivo. Inhibition studies of LF have documented that in vitro LF proteolysis of peptide was not hindered by the addition of protease inhibitors, bestatin and lysine CMK, at concentrations ranging from 50 μM to 1 mM, but that the same inhibitors at the concentration of 200 μM protected the cultured macrophage from lysis by *anthrax* LeTx [14]. The authors interpreted these results as a protective effect by these inhibitors observed in toxin-challenged cultured macrophage might not be due to direct inhibition of enzymatic activity but rather to some other event in the cytolytic cascade. Based on our results, it could be also explained that LF after endocytosis undertakes an active site rearrangement displaying a different inhibition profile compared to the enzyme before pH alteration. It should be noted that the endocytosis of anthrax toxin is mediated by heptameric PA complexes [25,26]. Therefore, it remains to be investigated about the effect of PA on catalysis of LF during endocytosis.

Taken together, we suggest that our observation should be taken into account when designing LF inhibitors. Therefore, elucidation of LF substrate hydrolysis in terms of in vivo may be important for rational designing of potent and selective inhibitors to prevent both binding and catalysis of enzyme to its substrate. Of course, it is difficult to inhibit protein–protein interactions by small molecules, because the inhibitor has to be adapted to the structural specificities involved in the binding of the two partners.

Although further studies are required, pH dependence of LF activity strongly suggests that it induces a subtle structural rearrangement to provide its own catalytic properties and substrate selectivity. We are currently pursuing the development of high-throughput analysis of LF activity using the native substrate to determine the kinetic parameters, such as K_m, K_{cat}, and V_{max}.

Acknowledgments

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References

[1] S.H. Leppla, Production and purification of anthrax toxin, in: Methods in Enzymology, vol. 165, Academic Press, 1988, pp. 103–116.
[2] M. Mock, A. Fouet, Annu. Rev. Microbiol. 55 (2001) 647–671.
[3] N. Morton, M.D. Swartz, N. Engl. J. Med. 345 (2001) 1621–1626.
[4] D.M. Gill, Bacterial Toxins and Cell Membranes, Academic Press, New York, 1978, pp. 291–332.
[5] H.M. Koo, M. VanBrocklin, M.J. McWilliams, S.H. Leppla, N.S. Duesbery, G.F. Woude, Proc. Natl. Acad. Sci. USA 99 (2002) 3052–3057.
[6] S.G. Popov, R. Villasmil, J. Bernardi, E. Grene, J. Cardwell, T. Popova, A. Wu, D. Alibek, C. Bailey, K. Alibek, FEBS Lett. 527 (2002) 211–215.
[7] S.G. Popov, R. Villasmil, J. Bernardi, E. Grene, J. Cardwell, A. Wu, D. Alibek, C. Bailey, K. Alibek, Biochem. Biophys. Res. Commun. 283 (2002) 349–355.
[8] R. Pellizzari, C. Guidi-Rontani, G. Vitale, M. Mock, C. Montecucco, FEBS Lett. 462 (1999) 199–204.
[9] J.M. Park, F.R. Greten, Z.W. Li, M. Karin, Science 297 (2002) 2048–2051.
[10] N.S. Duesbery, C.P. Webb, S.H. Leppla, V.M. Gordon, K.R. Klimpel, T.D. Copeland, N.G. Ahn, M.K. Oskarsson, K. Fukasawa, K.D. Paull, G.F. Vande Woude, Science 280 (1998) 734–737.
[11] G. Vitale, L. Bernardi, G. Napolitani, M. Mock, C. Montecucco, Biochem. J. 352 (2000) 739–745.
[12] G. Vitale, R. Pellizzari, C. Recchi, G. Napolitani, M. Mock, C. Montecucco, Biochem. Biophys. Res. Commun. 248 (1998) 706–711.
[13] N.S. Duesbery, J. Resau, C.P. Webb, S. Koochekpour, H.M. Koo, S.H. Leppla, W. Vande Wonde, Proc. Natl. Acad. Sci. USA 98 (2001) 4089–4094.
[14] S.E. Hammond, P.C. Hanna, Infect. Immun. 66 (1998) 2374–2378.
[15] R.T. Cummings, S.P. Salowe, B.R. Cunningham, J. Wiltsie, Y.W. Park, L.M. Sonatore, D. Wisniewski, C.M. Douglas, J.D. Hermes, E.M. Scolnick, Proc. Natl. Acad. Sci. USA 99 (2002) 6603–6606.
[16] F. Tonello, P. Ascenzi, C. Montecucco, J. Biol. Chem. 278 (2003) 40075–40078.
[17] A.D. Pannifer, T.Y. Wong, R. Schwarzenbacher, M. Renatus, C. Petosa, J. Bienkowska, D.B. Lacy, R.J. Collier, S. Park, S.H. Leppla, P. Hanna, R.C. Liddington, Nature 414 (2001) 229–233.
[18] A.P. Chopra, S.A. Boone, X. Liang, N.S. Duesbery, J. Biol. Chem. 278 (2003) 9402–9406.
[19] J. Kim, Y.M. Kim, B.S. Koo, Y.G. Chae, M.Y. Yoon, Protein Expr. Purif. 30 (2003) 293–300.
[20] S. Park, S.H. Leppla, Protein Expr. Purif. 18 (2000) 293–302.
[21] M. Mock, B.P. Roques, Proc. Natl. Acad. Sci. USA 99 (2002) 6527–6529.
[22] C. Radha, P. Salotra, R. Bhat, R. Bhatnagar, J. Biotechnol. 50 (1996) 235–242.
[23] K.R. Klimpel, N. Arora, S.H. Leppla, Mol. Microbiol. 13 (1994) 1093–1110.
[24] R. Bhatnagar, Y. Singh, S.H. Leppla, A.M. Friedlander, Infect. Immun. 57 (1989) 2107–2117.
[25] R.K. Klimpel, S.S. Molloy, G. Thomas, S.H. Leppla, Proc. Natl. Acad. Sci. USA 89 (1992) 10277–10281.
[26] R.O. Blaustein, T.M. Koehler, R.J. Collier, A. Finkelstein, Proc. Natl. Acad. Sci. USA 86 (1989) 2209–2213.
[27] A.M. Friedlander, J. Biol. Chem. 261 (1986) 7123–7126.
[28] P. Gupta, S. Singh, A. Tiwari, R. Bhat, R. Bhatnagar, Biochem. Biophys. Res. Commun. 284 (2001) 568–573.
[29] F. Cornille, L. Martin, C. Lenoir, D. Cussac, B.P. Roques, M. Fournie-Zaluski, J. Biol. Chem. 272 (1997) 3459–3464.
[30] B.P. Roques, F. Noble, V. Dauge, M.C. Fournie-Zaluski, A. Beaumont, Pharmacol. Rev. 45 (1993) 87–146.