ART & TECHNOLOGY PUBLISHING PTE. LTD.

Gene Science and Engineering

2023 Volume 3, Issue 1

ISSN: 2529-7651

Capturing the Active Transition States of LPS-Sensitive Protease Precursors in Autocatalytic Activation

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Abstract

Hemolymph coagulation in horseshoe crabs is triggered by the autocatalytic activation of lipopolysaccharide (LPS)-sensitive serine protease zymogen factor C through its transition state (factor C*). However, the existence of factor C* is only speculative, and it remains unknown whether the autocatalytic cleavage of the Phe737-Ile738 bond (the F737 site) of factor C* required for the conversion to an active form α-factor C occurs intramolecularly or intermolecularly. We show that the F737 site of a catalytic Ser941-deficient mutant of factor C is cleaved by an F737 site-uncleavable mutant in the presence of LPS. These data clearly indicate the existence of factor C* without cleavage of the F737 site. We also found the following facts. Firstly, the autocatalytic cleavage at the F737 site of factor C* occurs intermolecularly on the LPS surface. Secondly, factor C* does not exhibit intrinsic chymotryptic activity against the F737 site during the autocatalytic activation. Thirdly, LPS is required not only to complete the substrate-binding site and oxyanion hole of factor C* but also to allow the F737 site to be cleaved.

Keywords

Horseshoe crab
Lipopolysaccharide (LPS)
Serine protease zymogen
Autocatalytic activation
Transition state

1. Introduction

Horseshoe crabs belong to the subphylum Chelicerata, which includes spiders, scorpions, and mites, and not to the trilobite subphylum, which flourished during the Paleozoic era. In the Burgess Shale fauna of North America (ca. 505 million years ago), which consists of arthropod fossil assemblages, chelicerates such as *Sanctacaris uncata* and *Habelia optata* have already appeared together with trilobites. Extant species include *Tachypleus tridentatus* in Japan, *Limulus polyphemus* on the eastern coast of North America, and *T. gigas* and *Carcinoscorpius rotundicauda* in addition to *T. tridentatus* on the southeast coast of Asia. *T. tridentatus* is found on the southeastern coast of Asia.

The immune system of the horseshoe crab is characterized by a high sensitivity to lipopolysaccharide

(LPS), a component of the cell wall of gram-negative bacteria, and the fluid coagulation reaction elicited by it. Granule cells in horseshoe crab fluid are sensitive to LPS and release coagulation factors stored in their granules [1]. Coagulation factors comprise four serine protease precursors (Factor C, Factor G, Factor B, and coagulation enzyme precursors), and a coagulation protein precursor, coagulogen, which triggers a chain reaction of activation by limited degradation (cascade reaction) as shown in **Figure 1**. Factor C binds to LPS via a triplet sequence (-Arg^{36}-Trp^{37}-Arg^{38}-) in its N-terminal domain [2] and undergoes autocatalytic limited degradation to become Factor α-C, which in turn converts factor B into the active form. Active factor B activates coagulogen precursors to coagulases, and coagulogen is converted by coagulases to coagulins, which gelatinize.

Factor C also functions as an LPS receptor localized to the plasma membrane of granulocytes and is presumed to activate protease-dependent G protein-coupled receptors [3]. Factor G, on the other hand, interacts with β-1,3-D-glucan (BDG), a cell wall component of fungi and seaweeds, and activates it in an autocatalytic manner to activate coagulase precursors without the intervention of factor B, which is also shown in **Figure 1**. BDGs and peptidoglycans do not stimulate granulocytes to release coagulation factors [3].

Wang *et al.* demonstrated that N-linked carbohydrate chains are essential for the folding of factor C in the Drosophila S2 cell system [4]. Subsequently, we prepared recombinant factor C with a fixed-size N-linked sugar chain ($Man_5GlcNAc_2$) using mammalian cells (HEK293S mutant strain). However, the resulting recombinant factor C showed LPS-binding activity comparable to that of natural factor C but was not autocatalytically active. Amino acid sequence analysis showed that three vector-derived amino acid residues were added to the N-terminal Arg^1 residue, and when the pro-peptide of the original factor C was inserted on the amino-terminal side of Arg^1, the pro-peptide was successfully processed in cultured cells, and the recombinant factor C exhibited LPS-binding activity comparable to that of the natural factor C [5]. Furthermore, it was found that Arg^1 is essential for autocatalytic activation and that the distance between Arg^1 and the LPS-binding sequence is important for autocatalytic activation.

We postulate a transition state (factor C*) that has not undergone limited degradation but has an active conformation to explain the LPS-dependent

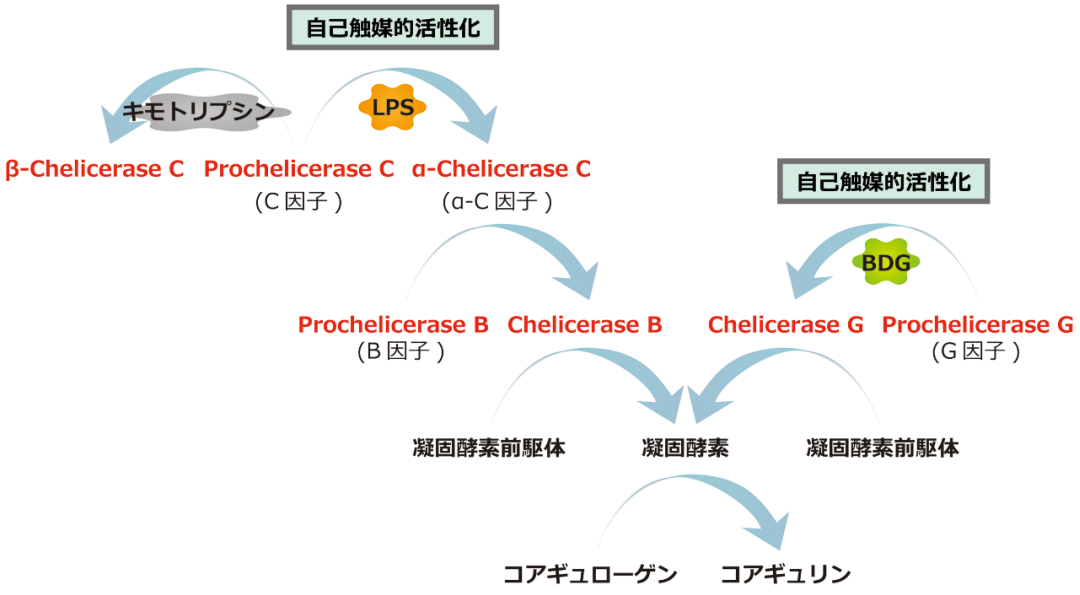

Figure 1. Cascade reaction of horseshoe crab fluid coagulation. New coagulation factor designations are shown in red.

autocatalytic activation mechanism of factor C. Judging from the sequence homology, the factors α-C belong to the tryptic family, favoring Arg or Lys residues at the cleavage position of the substrate (P1 site) and, in fact, specifically cleave synthetic peptide substrates with Arg at the P1 site. However, as the conversion of factors C to factors α-C via autocatalytic activation involves limited degradation of the F737 moiety as presented in **Figure 2** [6], the convenient assumption that factors C* have intrinsic chymotrypsin-like activity to cleave the F737 moiety was also necessary. Furthermore, whether the F737 site of the factor C* is cleaved intramolecularly or intermolecularly by the factor C* was an open question. To resolve these concerns, a detailed study was carried out using mutants of the factor C [7].

2. Mechanism of activation of serine protease precursors via limited degradation

The activation mechanism of the serine protease precursor is explained by a model of activation via trypsin-mediated limiting degradation of chymotrypsinogen. That is, when the -Arg^{15}-Ile^{16}- peptide bond of bovine chymotrypsinogen is limitedly degraded by trypsin, the newly generated Ile^{16}-Val^{17}-Asn^{18}-Gly^{19}- peptide sequence of bovine chymotrypsinogen is folded and penetrates the hydrophobic hole called the activation pocket of chymotrypsinogen, where it is separated from the α-amino group of Ile^{16} by a peptide sequence called -Asn^{18}-Gly^{19}-. The α-amino group of Ile^{16} and the β-carboxyl group of the Asp^{194} side chain inside the molecule form a salt bridge. This induces a conformational change around the catalytic site (a triad of His^{57}, Asp^{102}, and Ser^{195}), completing the substrate binding site (for Arg and Lys side chains) and the oxyanion hole (site for temporary stabilization of the tetrahedral intermediate of the substrate-enzyme complex). In other words, the essence of serine protease precursor activation is not the limited degradation of -Arg^{15}-Ile^{16}- but the conformational change induced by intramolecular salt bridge formation. Therefore, even without limited degradation, the precursor should be converted to the active form if it has the same action as salt bridge formation.

Factor C is a double-stranded precursor consisting of H and L chains, with the L chain containing the protease domain as shown in **Figure 2**. Following the chymotrypsinogen activation model, the autocatalytic activation of factor C* was estimated to be the same as that of the chymotrypsinogen activation model. The factor C* in the transition state upon interaction with LPS autocatalytically and limitedly degrades -Phe^{737}-Ile^{738}- (F737 site), which corresponds to -Arg^{15}-Ile^{16}-, intra- or intermolecularly. As a result, the peptide sequence Ile^{738}-Trp^{739}-Asn^{740}-Gly^{741}- folds and sneaks into the activation pocket, where the α-amino group of Ile^{738} forms a salt bridge with the side-chain β-carboxyl group of Asp^{940}, which corresponds to Asp^{194} and is converted to factor α-C. Experimentally, the conversion of Factor C to Factor α-C has been confirmed by, (1) Western blotting with an antibody against the B-chain to show that the B-chain is released from the L-chain upon cleavage of the F737 site, and (2) measuring the amidase activity against a synthetic peptide substrate.

3. Autocatalytic limiting degradation does not require the assumption of the intrinsic chymotrypsin-like activity of the factor C*

Since the S941A mutant, in which the Ser^{941} residue of factor C corresponding to the catalytic residue Ser^{195} of the serine protease is replaced by Ala, does not induce LPS-dependent autocatalytic activation, it is clear that Ser^{941} also functions as the catalytic site in factors C*. Therefore, to confirm the intrinsic chymotrypsin-like activity of the factor C*, we replaced Phe^{737} at the F737 site with Ala (F737A mutant) or Glu (F737E mutant). Contrary to expectations, the F737A and

F737E mutants induced LPS-dependent autocatalytic activation of the F737A and F737E mutants, as in the wild type. This means that Phe^{737} at the P1 site is not important for substrate recognition during the limited degradation of the F737 site by the factor C*.

In contrast, the F737P mutant, in which Phe^{737} was replaced by a Pro residue, did not show LPS-dependent limiting degradation. The Pro residue is an amino acid that breaks secondary structures such as the α-helix and β-sheet, and the conformational change around the F737 moiety is not responsible for the conformational change. The conformational change around the F737 site may be essential for the limited degradation by the factor C*. These experimental results indicate that autocatalytic limiting degradation of the F737 site does not require the assumption of the intrinsic chymotrypsin-like activity of the factor C*.

4. Factor C* capture and intermolecular limited degradation of the F737 moiety

The S941A mutant loses the catalytic residue Ser^{941}, but the F737 site is normal. On the other hand, Ser^{941} in the F737P mutant is normal, but the F737 site is not limitedly degraded. If the two mutants are mixed in the presence of LPS, the F737P mutant takes a transition state (F737P*) and intermolecular limited degradation occurs, the F737 site in the S941A mutant should be limitedly degraded. When the S941A:F737P molar ratio was mixed at 50:1, 50:2, and 50:5 in the presence of LPS, and incubated for a certain time, the F737 site of the S941A mutant was cleaved in proportion to the amount of F737P, demonstrating the presence of the transition state F737P* (factor C*).

To further confirm the intermolecular limited resolution by the factor C*, it was necessary to tag factor C with a labeling tag. However, the introduction of a His-tag at the N- or C-terminus of factor C did not affect LPS affinity but inhibited autocatalytic activation. However, in **Figure 2**, factor PA-C, in which a PA-tag (a 10-residue peptide derived from podoplanin) was introduced into the B strand of factor C, was activated in the presence of LPS. The PA-tag is considered to be less likely to cause functional impairment of the introduced protein due to its ring-like structure. Therefore, a PA-S941A mutant was prepared by introducing a PA-tag into the B strand of the S941A mutant, and when the PA-S941A mutant and the F737P mutant were mixed 1:1 in the presence of LPS, the F737 moiety of the PA-S941A mutant was limitedly degraded by F737P* and the release of the

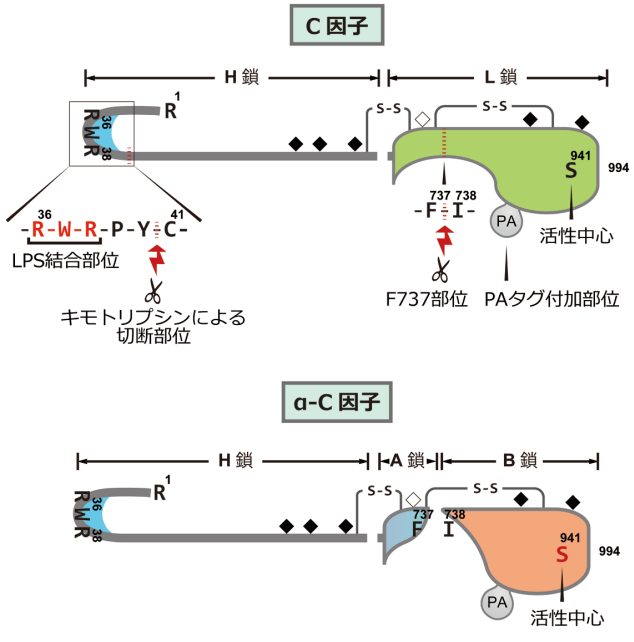

Figure 2. Model diagram of factor C and factor α-C. Factor C binds to LPS via a triplet sequence (-R^{36}-W^{37}-R^{38}-) localized on the N-terminal side and undergoes a limited degradation of the -F737-I738- (F737 site) by an autocatalytic activation reaction, resulting in conversion to active factor α-C [5]. In this process, the L chain is cleaved to produce the A and B chains. The F737 moiety of Factor C is also cleaved by chymotrypsin to form factor β-C, which has amidase activity against peptide substrates. However, factor β-C cannot activate Factor B because the secondary -Y^{40}-C^{41}- is also cleaved by chymotrypsin [8].

B strand was confirmed by PA-tag antibody. These results demonstrate that the factor C* is capable of intermolecularly limiting the degradation of the F737 moiety. It should be noted that F737P* is a mutant in which the F737 moiety is not originally cleaved, and this does not directly prove that the factor C* does not cleave the F737 moiety intramolecularly.

5. Intermolecular reactions between factors C* require a scaffold LPS

Factors RE-C, in which the two Arg residues of the LPS-binding motif (-Arg^{36}-Trp^{37}-Arg^{38}-) of the factor C were replaced with Glu residues, lose their LPS-binding activity and do not induce LPS-dependent autocatalytic activation [3]. Therefore, a RE-S941A mutant lacking LPS-binding activity was created. In the presence of LPS, the RE-S941A mutant was mixed 1:1 with the F737P mutant, but the F737 site of the RE-S941A mutant was not degraded. This suggests that for limited degradation of the intermolecular F737 moiety by the factor C*, the substrate-side factor C must also be bound to the LPS. The binding of the substrate-side factor C to LPS may convert the F737 moiety to a conformation that is more susceptible to limited degradation.

6. Renaming of horseshoe crab coagulation factors

Recently, Factor B was also found to be an LPS-binding protein [8]. On the other hand, complement C3 factor, complement B factor and complement lectin are present in the plasma of horseshoe crabs [9,10]. Interestingly, Factor C forms a complex with complement C3 factor and plays an important role in the initial process of complement activation [11]. Furthermore, a homolog of the mite factor C has been found and it is involved in the complement system [12]. Thus, due to the overlap in name and function between the horseshoe crab coagulation factor and the complement system factor, the horseshoe crab coagulation factor was renamed [7]. In reference to the taxonomic names of the subfamily Sciurinae, the C, B, and G factors were designated Prochelicerase C, Prochelicerase B, and Prochelicerase G, respectively, with the active form excluding Pro (**Figure 1**). For Prochelicerase C, those activated autocatalytically were designated α-chelicerase C, and those activated by chymotrypsin were designated β-chelicerase C. It should be noted that β-chelicerase C shows amidase activity equivalent to α-chelicerase C for synthetic peptide substrates, but the -Tyr^{40}-Cys^{41}- peptide bonds near the LPS-binding motif are not active in β-chelicerase C. The bonds in the vicinity of the LPS-binding motif are cleaved by chymotrypsin, resulting in the loss of LPS-binding properties, and do not activate factor B (Prochelicerase B), as shown in **Figure 2** [8].

7. Conclusion

It was a great achievement to show the existence of a

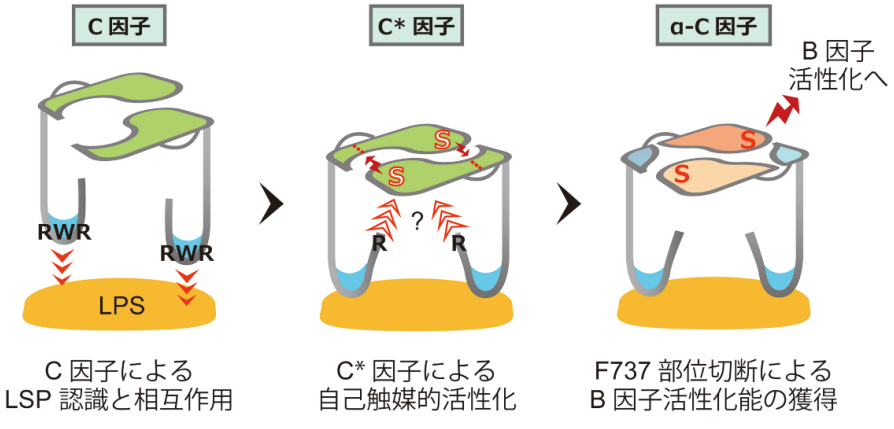

Figure 3. Model diagram of the autocatalytic activation of Factor C in the presence of LPS. Factor C bound to LPS via a triplet sequence forms a complex of factors C* in the transition state. Factor C* is converted to factor α-C by intermolecular cleavage of the F737 moiety, which activates the next precursor in the cascade, Factor B.

transition state factor C* by using the factor C mutant, as presented in **Figure 3**. In addition, the following features of the factor C* were elucidated. Firstly, autocatalytic limited degradation of the F737 moiety is an intermolecular reaction between factors C*. Plus, it is not necessary to assume the intrinsic chymotrypsin-like activity of factor C* for the limited degradation of the F737 site. Additionally, LPS binding not only completes the substrate binding site and oxyanion hole of the factor C* but also induces conformational changes around the F737 site. In future research, recombinant proteins of all coagulation factors should be prepared and the molecular mechanism of the coagulation cascade should be analyzed in detail.

Disclosure statement

The authors declare no conflicts of interest.

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