Head-to-Tail Polymerization of Coagulin, a Clottable Protein of the Horseshoe Crab*

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A clottable protein coagulogen of the horseshoe crab Tachypleus tridentatus is proteolytically converted into an insoluble coagulin gel through non-covalent self-polymerization. Here we identified binding sites for the polymerization. A tryptic fragment, derived from the coagulin polymer chemically cross-linked by a bifunctional cross-linker, was isolated. Amino acid sequence analysis indicated that the fragment consists of two peptides cross-linked between Lys30 and Lys136. The two lysine residues are oppositely located at the head and tail regions of the elongated molecule separated by a much greater distance than the length of the cross-linker, which suggests that the cross-linking occurs intermolecularly. Based on the x-ray structural analysis, exposure of a hydrophobic cove on the head in response to the release of peptide C has been postulated (Bergner, A., Oganessyan, V., Muta, T., Iwanaga, S., Typke, D., Huber, J. R., and Bode, W. (1996) EMBO J. 15, 6789–6797). An octapeptide containing Tyr136, which occupies the tail end of coagulin, was found to inhibit the polymerization. Replacement of Tyr136 of the peptide with Ala resulted in loss of the inhibitory activity. These results indicate that the polymerization of coagulin proceeds through the interaction between the newly exposed hydrophobic cove on the head and the wedge-shaped hydrophobic tail.

Hemolymph coagulation in horseshoe crab is induced by lipopolysaccharides (LPS) of Gram-negative bacteria. This response is very important for the host defense, which involves the engulfment of invading microorganisms, and also for prevention of leakage of hemolymph (1–4). The immobilized invaders could be recognized by several lectins and subsequently killed by antimicrobial substances released from hemocytes (4–6). The LPS-mediated coagulation cascade involves three serine protease zymogens, including factor C, factor B, and the proclotting enzyme, and a clottable protein coagulogen (1–4). Factor C is a biosensor that responds to LPS. In the presence of LPS, factor C is autocatalytically converted to its active form. The activated factor C catalyzes the activation of factor B, and, in turn, the active form of factor B converts the proclotting enzyme to the clotting enzyme (1–4). The clotting enzyme cleaves coagulogen of 175 amino acid residues at two sites, yielding a fragment called peptide C (Thr19–Arg46) and the resulting coagulin, which consists of the NH2-terminal A-chain (Ala1–Arg18) and the COOH-terminal B-chain (Gly47–Phe175), connected by two disulfide bridges, forms an insoluble gel by self-polymerization (7–9). Crystal structural analysis of coagulogen revealed an elongated molecule (approximate dimensions, 60 × 30 × 20 Å) with a topological similarity to nerve growth factor (10, 11). The structural analysis suggested a possible polymerization mechanism, in which the release of the helical peptide C would expose a hydrophobic cove on the “head,” which interacts with the hydrophobic edge or “tail” of a second molecule, resulting in the formation of coagulin gel.

Here, by using chemical cross-linkers and synthetic peptides, we obtained evidence that the polymerization of coagulin proceeds through the interaction between the hydrophobic cove on the head and the hydrophobic tail.

EXPERIMENTAL PROCEDURES

Materials—Coagulogen (7–9) and the clotting enzyme (12) were prepared from hemocyte lysates of Tachypleus tridentatus as described. Homobifunctional cross-linkers, disuccinimidyl suberate (DSS), disuccinimidyl glutarate (DSG), and disuccinimidyl tartrate (DST) were obtained from Pierce. TPCK-trypsin was obtained from Worthington.

Cross-linking—Coagulogen (1 mg/ml in 20 mM HEPES, pH 8.0) was incubated with the clotting enzyme at 37 °C for 1 h (enzyme/substrate = 1/38, w/w). The resulting coagulin gel was dissolved by 10-fold dilution with the same buffer to a final concentration of 0.1 mg/ml, and no clot formation was observed under these conditions. A cross-linker in dimethylformamide was added to the coagulin solution to give a final concentration of 0.2 mM, and the solution was further incubated at 25 °C in a water bath. The reaction was stopped by adding 1 M Tris-HCl, pH 8.0, to give a final concentration of 0.1x, followed by incubation at 25 °C for 15 min. Cross-linked coagulin was treated with 10% trichloroacetic acid and the resulting precipitate was used for SDS-PAGE and tryptic digestion. As a negative control, cross-linking experiments for coagulogen were carried out under the same conditions. SDS-PAGE was performed in 15% slab gels under reducing conditions, according to the procedure described by Laemmli (13). The gels were stained with Coomassie Brilliant Blue R-250. The reference proteins were phosphorylase b (Mr = 94,000), bovine serum albumin (Mr = 67,000), ovalbumin (Mr = 35297
43,000, carbonic anhydrase (Mₐ = 30,000), soybean trypsin inhibitor (Mₐ = 20,000) and α-lactalbumin (Mₐ = 14,400).

Separation of Tryptic Peptides of Cross-linked Coagulin—The precipitate of cross-linked coagulin (250 μg) was dissolved in 0.4 M NH₄HCO₃ containing 8% H₂O. It was reduced and S-alkylated with iodoacetamide (14), then digested with TPC(trypsin (enzyme/substrate = 1:20, w/w). The resulting peptides were separated by reverse-phase HPLC on a column of Cosmosil 5C₁₈-MS (20 × 150 mm, Nacalai Tesque, Kyoto, Japan). Peptides were eluted with a linear gradient of 0–80% acetonitrile in 0.06% trifluoroacetic acid at a flow rate of 0.2 ml/min. Absorbance was monitored at 210 nm. Acidic peptide sequence analysis was performed with the Applied Biosystems gas-phase sequencer model 470A.

Peptide Synthesis—The oligopeptides, Ala-Gly-Tyr-Asn and Ser-Ala-Gly-Tyr-Asn-Gly, were synthesized by the manual solid phase method using t-butoxycarbonyl amino acids. Coupling reactions were carried out with 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate in the presence of 1-hydroxybenzotriazole in a mixture of N,N-dimethylformamide and N,N-dimethylethanolamine (1:2, v/v) for 30 min. Peptides were liberated from the resin by treatment with anhydrous liquid HF containing 10% p-cresol at 0 °C for 1 h. Syntheses of Asn-Ser-Ala-Gly-Tyr-Asn-Gly, Arg-Ser-Ala-Gly-Phe-Ala-Gly, Arg-Ser-Ala-Gly-Asn-Gly-Arg, and Cys (acetamidomethyl)-Asn-Ser-Ala-Gly-Tyr-Asn-Gly-Ary (16). The purity of the peptides was verified by analytical reverse-phase HPLC (0.4 × 25 cm, LiChrospher 100 RP-18).

Competition of Synthetic Peptides for Coagulin Polymerization—Coagulin (2 mg/ml in 20 mM Tris-HCl, pH 8.0) was treated with TPC(trypsin (enzyme/substrate = 1/100, w/w) in the presence and absence of synthetic peptides, respectively. The time course of gel formation was monitored by measuring the scattered light at 339 nm with a time-resolved phototransformer. The polymerization was dose-dependently inhibited by the addition of the octapeptide (Fig. 3A). The re-plot of the initial velocities of polymerization versus the concentrations of the octapeptide showed a 50% inhibitory concentration (IC₅₀) of 2.3 mM (Fig. 3B). When Tyr₃⁵⁶ of the octapeptide was replaced with Phe, the inhibitory effect was observed with a 2.5-fold higher IC₅₀ of 5.7 mM. However, replacement with Ala demonstrated no inhibition on the polymerization at 5.0 mM (Table II). Inhibitory experiments at higher concentrations of the octapeptide showed a 50% inhibitory concentration (IC₅₀) of 2.3 mM (Fig. 3B). When Tyr₃⁵⁶ of the octapeptide was replaced with Phe, the inhibitory effect was observed with a 2.5-fold higher IC₅₀ of 5.7 mM. However, replacement with Ala demonstrated no inhibition on the polymerization at 5.0 mM (Table II). Inhibitory experiments at higher

Fig. 1 SDS-PAGE of cross-linked coagulin. Lane 1, purified coagulin; lane 2, coagulin in the presence of DSS; lane 3, cross-linked coagulin.
concentrations of the Ala136-containing peptide could not be carried out due to the low solubility of the peptide. The hexapeptide and tetrapeptide containing Tyr136 had no effect, suggesting that a proper conformation around Tyr136 is required for the interaction. To mimic the β-turn of the original structure, an undecapeptide was synthesized with Cys replacing Ser131, and a disulfide bridge was formed, linking with Cys140. The undecapeptide exhibited a slightly lower IC50 than the octapeptide containing Tyr136 (Table II).

The hydrophobic tail portion of coagulin seems to have the same conformation as that of coagulogen, given that the cleavage and removal of the helical peptide C consistently occurs at the opposite side of the elongated molecule (10). The tail region of coagulogen, therefore, could interact with the hydrophobic head of coagulin to form a heterodimer. To investigate this hypothesis, the interaction of coagulogen and the immobilized coagulin on a sensor chip was determined by surface plasmon resonance. Coagulogen at various concentrations was passed over the immobilized coagulin. Fig. 4 shows the sensorgrams of association and dissociation reactions as a relative response against time. When coagulogen rather than coagulin was immobilized on the sensor chip, no interaction was observed between the coagulogen molecules (data not shown). Analysis of the association and dissociation phases of the sensorgrams revealed an association rate constant \( k_a = 1.0 \times 10^2 \) M\(^{-1}\) s\(^{-1}\) and a dissociation rate constant \( k_d = 6.0 \times 10^{-4} \) s\(^{-1}\), and,

**TABLE I**

| Cycle no. | Phenylthiohydantoin amino acids (yield, pmol) |
|-----------|----------------------------------------------|
| 1         | Cys(nq) \(^a\) Leu(28)                       |
| 2         | Tyr(48) Val(33)                              |
| 3         | Asn(13) Thr(3)                               |
| 4         | Phe(46) Tyr(22)                              |
| 5         | Pro(47) Asn(6)                               |
| 6         | Pro(44) Leu(18)                              |
| 7         | Phe(42) Glu(11)                              |
| 8         | Thr(2) X                                     |
| 9         | His(13) Asp(9)                               |
| 10        | Phe(26) Gly(10)                              |
| 11        | X Phe(28)                                    |
| 12        | Ser(nq) Leu(23)                              |
| 13        | Glu(11) Cys(nq)                              |
| 14        | Cys(nq) Glu(10)                              |
| 15        | Pro(12) Ser(nq)                              |
| 16        | Val(14) Phe(7)                               |
| 17        | Ser(nq) Arg(3)                               |
| 18        | Thr(nq)                                      |
| 19        | Arg(3)                                       |

Position CYNFPFPPTHKSECPVSTR (75–93)  
LVTYNLEKGFLCESFR (149–165)  

**TABLE II**

| Sequence | IC50, 50% inhibitory concentration of the initial velocity |
|----------|----------------------------------------------------------|
| NSAGYNGR | 2.3                                                      |
| NSAGPGNR | 5.7                                                      |
| NSAGANGR | —                                                       |
| SAGYNG   | —                                                       |
| AGYN     | —                                                       |
| CNSAGYNGRC | 1.8                                                  |

\(^a\) —, no inhibition at 5 mM.
consequently, \( K_a = 1.7 \times 10^8 \text{ m}^{-1} \). Horseshoe crab hemolymph contains high concentrations of \( \text{Ca}^{2+} \) and \( \text{Mg}^{2+} \): 9.9 and 46 mM, respectively (19). \( \text{CaCl}_2 \) or \( \text{MgCl}_2 \) at 10 mM, however, had no effect on the interaction between coagulogen and the immobilized coagulin.

**DISCUSSION**

Hemolymph coagulation in horseshoe crab is thought to be analogous to the vertebrate-clotting cascade. Coagulogen, a functional homologue of fibrinogen, is proteolytically converted into coagulin to form an insoluble gel through non-covalent self-polymerization (7–9). The two proteins, however, are quite different in molecular size and amino acid sequence. Interestingly, a structural homologue of fibrinogen has recently been identified in horseshoe crab plasma, although it functions as a non-self-recognizing protein (20). The crystal structure of coagulogen suggested a possible polymerization mechanism, by which, it was theorized, the removal of the peptide C would expose a hydrophobic cove on the head of one coagulin, thereby providing a new interaction site for the hydrophobic surface of the second molecule (10). We herein proved this hypothesis of the head-to-tail polymerization, by using chemical cross-linkers and synthetic peptides and by performing surface plasmon resonance analysis.

Fig. 5 shows a putative structural model of a coagulin dimer formed through head-to-tail interaction. Lys\(^{85}\) in one molecule and Lys\(^{156}\) in another molecule can be within 6.4 Å apart after the conversion from coagulogen to coagulin, as demonstrated by cross-linking experiments. An octapeptide, corresponding to the hydrophobic tail, inhibited the polymerization of coagulin with \( IC_{50} \) of 2.3 mM (Fig. 3 and Table II). The replacement of Tyr\(^{136}\) of the octapeptide with Phe increased the \( IC_{50} \) value by 2.5-fold, whereas replacement with Ala showed no inhibitory activity, suggesting the importance of Tyr\(^{136}\), particularly for the aromatic ring, in obtaining the proper affinity for polymerization. Furthermore, a hexapeptide containing Tyr\(^{136}\) also lost the inhibitory activity, indicating that the shorter peptide does not have sufficient ability to hold the conformation required for the interaction. Several synthetic peptides corresponding to the NH\(_2\)-terminal regions of the fibrin \( \alpha \) - and \( \beta \)-chains have been reported to prevent the polymerization of fibrin monomers, and a peptide of Gly-Pro-Arg-Pro was reported to bind to fibrinogen (21, 22) and to the fragment D with \( K_a = 0.5 \times 10^5 \text{ m}^{-1} \) (23, 24).

The surface plasmon resonance analysis revealed that the
interaction of coagulogen with the immobilized coagulin, possibly through the tail of coagulogen and the head of coagulin, with $K_0 = 1.7 \times 10^6 \text{m}^{-1}$. Not only the coagulin monomer but also coagulogen could be incorporated into a coagulin fiber, then converted to coagulin by the clotting enzyme, leading to the extension of the fiber. Relative to this finding, if serine proteases in the coagulation cascade are scavenged in vivo by the horseshoe crab serine protease inhibitors (25–27), coagulogen could regulate the extension of the fiber to bind to the terminus. Electron microscopy showed that coagulin fibers have a tendency to aggregate laterally to form a thicker fiber with a diameter of about 100 Å, probably through other hydrophobic patches on the surface of coagulogen (10). The resulting thick fibers may form a reticulum, catching invading bacteria and substances from outside. The binding site(s) for such side-to-side interaction remains to be determined. A hypothetical scheme of the gel formation is shown in Fig. 6.

In the vertebrate coagulation system, factor XIIa, a plasma transglutaminase, covalently cross-links fibrin to form a stable cross-linked fibrin with itself or with other proteins, which is essential for normal homeostasis and wound healing (28). In horseshoe crab hemolymph, however, a transglutaminase is present in the cytosol of hemocytes but not in the plasma (29, 30). Furthermore, coagulogen and coagulin themselves are not substrates for the hemocyte transglutaminase; the proteins of 8.6 and 80 kDa present in hemocytes serve as substrates for the transglutaminase (4, 29). These proteins may participate in forming the reticulum structure of the coagulin gel.

Another protease cascade in invertebrates, the morphogenetic cascade for determining embryonic dorsal-ventral polarity in the fly Drosophila melanogaster, has been well characterized at the molecular level (31). The structural similarity of the target protein of the cascade, Drosophila toll-receptor ligand spätzle, to horseshoe crab coagulogen, as well as the sequence homology between the serine proteases of the two cascades, suggest that these two functionally different cascades may have a common origin (10, 11, 32, 33). The activation of Drosophila toll is also critical in the production of antibacterial and antifungal peptides as a response to microbial infection (34, 35). Although the molecular mechanism for the activation of toll by spätzle is not currently known, many membrane receptors are known to be activated through ligand-induced dimerization or oligomerization (36). Based on consideration of the polymerization process of coagulin, it seems possible that the ligand spätzle may also induce dimerization or oligomerization of Drosophila toll, leading to the activation of intercellular signaling (37). Ideally, the polymerization mechanism demonstrated here in coagulin will contribute to the body of knowledge concerning proteolysis-induced associations of proteins in various biological phenomena.

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