Effect of Plasmin Treatment on the Fibrin Gel Formation

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In order to clarify the effective regions that are substantially involved in the gelling process of plasma glycoprotein fibrinogen, we examined the aggregating properties of plasmin-treated fibrinogen, fragment-X. Two types of fragment-X were prepared by the digestion at 6°C and 37°C (fragment-XY and -XN, respectively). α C regions were cleaved thoroughly in both samples, but the amount of cleavage of B β N region differed between them (higher in the fragment-XN). Thrombin- and reptilase-catalyzed fibrin polymerizations were studied for both of the samples. Although the B-knob:b-hole interaction does neither work in the reptilase-catalyzed fibrin polymerization nor the α C- α C interaction is present, network formation proceeded in the fragment-XY with delayed polymerization. In the fragment-XN, protofibril formation occurred, but lateral aggregation did not. Mixing effect of intact fibrinogen showed that B β N region might play an important role in the lateral aggregation process cooperatively with α C-domain. .

Key words: fibrinogen, fibrin gel, lateral aggregation, fragment-X, plasmin

1. INTRODUCTION

Fibrinogen is a rod-shaped soluble plasma glycoprotein that plays a significant role in hemostasis and wound healing [1,2]. Its main function is the formation of three-dimensional network constructed of fibrin fibers, fibrin clot. Fibrinogen is a dimer composed of three polypeptide chains, A α , B β , and γ chains, (A α B β γ)₂. Molecular weight is 340 kDa, and the molecular length is 45 nm. The amino termini of six polypeptide chains (A α N, B β N, and γ N) come together in the central E region, while the carboxyl termini of them (α C, β C, and γ C) extend through the triple-stranded coiled-coil region outward forming the β C and γ C modules at the distal D regions. α C chains fold backward from the D region to the central E region [3,4]. Each α C chain consists of the highly mobile α C-connector (A α 221-391) and compact α C-domain (A α 392-610) [5].

Fibrin polymerization is the final and most important step of the blood coagulation cascade. It proceeds in a stepwise manner [6,7]. At the first stage, fibrin monomers converted from fibrinogen by thrombin assemble in a half-staggered manner to form double-stranded protofibrils. At the second stage, protofibrils aggregate laterally to form fibrin fibers and three-dimensional fibrin network. In these aggregating processes, removal of fibrinopeptide A (FpA) at the amino terminal end of A α chain by the serine protease thrombin results in the formation of protofibrils and is the trigger of succeeding process. The exposed A-knob by the removal of FpA binds to the a-hole located in the γ C module in the D region. Thrombin cleaves fibrinopeptides B (FpB) at the amino terminal ends of B β chains, too. However, the FpB release is very slow and is accomplished sequentially after the FpA release. It has been modelled that the α C-domains are involved in the process of lateral aggregation of protofibrils and

have an important role in the fibrin polymerization [8]. On the protofibril formation and the cleavage of FpB, the α C-domains dissociate from the central E region and interact with other α C-domains of adjacent protofibrils. Thus, the α C-domains have been considered to be the most significant factor for the lateral aggregation.

However, synthesized recombinant fibrinogen, A α 251 fibrinogen, that lacks the α C-domains, exhibited almost the same thrombin-catalyzed polymerization as the intact fibrinogen, although there were some discernible differences in the lateral aggregation [9]. Furthermore, reptilase (or bathroxobin), that is another enzyme for fibrinogen to cleave only FpB, results in the normal fibrin gel. Then, the cleavage of FpB might not be necessarily essential to originate the dissociation of α C-domain and their interaction. Bowley and Lord have reported that b-hole and binding between B-knob and b-hole per se have no influence on fibrin polymerization [10]. Because the interaction between the α C-domain and central E region through FpB and the interaction between α C-domains have been ascertained [11], the importance of the α C-domain is clear, but another substantial factor for polymerization should be involved especially in the lateral aggregation process.

Plasmin, which is a proteolytic enzyme for fibrinogen, has been used to prepare fibrinogen decomposed products [12]. The α C regions are cleaved from fibrinogen molecules in the early proteolytic process. Fragment thus prepared is called as fragment-X, and is used in the study of fibrin polymerization. Portion of B β 1-42 (in case of human fibrinogen) in the B β N region is easily cleaved succeeding to the cleavage of α C regions because B β 42Arg is the susceptible site to the proteolysis by plasmin [13], and it is probable that desB β 1-42 fragment-X is likely to contaminate the preparation of fragment-X. (In the present report, the

number of amino acid residue denotes the number for human fibrinogen, unless denoted.) Polymerization of fibrinogen lacking B β 1-42 is seriously impaired [14]. Then, the contribution of B β N region must be indispensable for the normal polymerization. This is in consistency with the existence of weak transient interactions within and between fibrin molecules mediated by B β N regions demonstrated by the laser tweezers based force spectroscopy [15]. However, it should be mentioned that essentially nothing is known about the binding sites or mechanisms involved in the lateral aggregation, or about branching, although both of these processes are essential for the functional fibrin clot [16].

In this report we studied the aggregating properties of plasmin-treated fibrinogen, fragment X, with different amount of cleavage of B β 1-42 in order to clarify the respective roles of α C and B β N regions. By adjusting the reaction conditions of plasmin, we could prepare two kinds of fragment-X with thorough cleavage of α C region and different amount of cleavage of B β 1-42.

2. EXPERIMENTAL

Materials Bovine fibrinogen (type IV) was purchased from Sigma-Aldrich Co. Thrombin and reptilase were obtained from Wako Pure Chemical and Fluka Bio Chemicals, respectively. Plasmin was purchased from ClabioChem. Fibrinogen was dialyzed against PBS (phosphate buffer saline) to remove citrate salts in the original sample. Concentration of fibrinogen was determined spectrophotometrically using the absorbance at 280 nm using an extinction coefficient of 1.5 mL/mg cm. Thus prepared stock solution was frozen by liquid N₂ and stored in the deep-freezer (-80 °C) until use. Thrombin and reptilase were also prepared similarly.

Preparation of fragment-X Fibrinogen dissolved in PBS (20 mg/mL) was incubated with plasmin (5 mU/mL) at 6 °C (fragment-XY) and 37 °C (fragment-XN). The reaction was stopped by the addition of AEBSF (protease inhibitor) at the incubation time of 9 hrs (fragment-XY) and 70 min (fragment-XN). Those digested sample solutions were passed through lysine sepharose 4B column to remove plasmin. The eluted solutions containing fragment-X were purified by GPC using Hiloal Superdex 200 column using tris-buffer solution (20 mM tris-HCl, pH 7.4, 300 mM NaCl). Thus purified fragment-X samples were characterized by SDS-PAGE, and dialyzed against TBS (20 mM tris-HCl, pH 7.4, 150 mM NaCl), frozen, and stored at -80 °C. According to the results of 1-D and 2-D SDS-PAGE, cleavage of α C and B β N regions were 100 and ca 50 % for fragment-XY and 100 and ca 90 % for fragment XN, respectively. A slight amount of B β chain of fragment-XN was hydrolyzed in the coiled-coil region, too.

Fibrinopeptide release Fibrinopeptides (FpA and FpB) release of fragment-X was examined. Fragment-X solution (1.2 μ M) was aliquoted at various elapsed time after the addition of thrombin (10 mU/mL), heated to stop the reaction, and centrifuged. Supernatant was collected and analyzed by reverse phase HPLC using ODS column (COSMOSIL 5C18 AR-II). Detection was done at 210 nm. Released amount at the reaction time of 24 hrs was taken as 100% of release.

Measurements Turbidity measurements were carried out by using UV/VIS spectrophotometer at the wave-

Fig. 1. Thrombin-catalyzed fibrin polymerization of intact fibrinogen, fragment-XY and fragment-XN determined by the turbidity at 350 nm with time. Concentration of fibrinogen and fragment-X is 3 nM, and that of thrombin is 20 mU/mL. Fragment-XN did not form gel even at the elapsed time of 5 hrs.

length of 350 nm and at 37 °C. Optical density OD was monitored in the time course of fibrin polymerization after the addition of thrombin or reptilase. Light scattering measurements were also employed to obtain the scattered light intensity and correlation function at the scattering angle of 30° [6]. Incident light source was Ar-ion laser (488.0 nm). Correlation functions were analyzed by the inverse Laplace transformation to determine the decay rate distribution. Decay rate corresponds to the particle dimension, whereas the scattered light intensity does to the magnitude of aggregation.

3. RESULTS AND DISCUSSION

Fibrinopeptide release of FpA in fragment-XY was almost the same as that of the intact fibrinogen, whereas that in fragment-XN was decreased definitely. In contrast, FpB release in fragment-XY was became lowered substantially, and that in fragment-XN was severely impaired and the FpB release was almost undetected (Figures are not shown). Due to the deletion of B β N region, an inhibition of thrombin binding occurred, because β wall, to which thrombin binds, is influenced by the deletion of B β N region in proximity to it [17]. The lowered FpB release has been observed also in A α 251 fibrinogen, where B β N region is not cleaved [9]. The observation of essentially the same fibrin polymerization as that in the intact fibrinogen suggests that α C-domain has a role in regulating the FpB release and the possible interaction of α C-domain with B β N. This picture is demonstrated clearly in the reptilase-catalyzed fibrin polymerization as described below.

Fig. 1 depicts the time course of optical density for the intact fibrinogen, fragment-XY, and fragment-XN at 37°C in PBS. Concentrations of fibrinogen and thrombin were 3 μ M and 20 mU/mL, respectively. 1 mM CaCl₂ was added. For the intact fibrinogen, after the lag time period with very slow and slight increase of OD, rapid increase of OD appears which corresponds to the gelation and lateral aggregation of protofibrils (networking fiber growth). This is the characteristic behavior of fibrin polymerization. In spite that the

polymerization in fragment-XY was only slightly delayed, fragment-XN showed essentially no rapid increase and lateral aggregation did not occur. No occurrence of lateral aggregation in fragment-XN was ascertained by the correlation function measurement, too. Because the cleavage of α C region results in only a delay of the fibrin polymerization [9], these unique results suggest the important role of B β N region. The deletion of B β N region causes the absence of FpB and no interaction between B-knob and b-hole. The observed polymerization behaviors could be attributed to the difference in the magnitude of B β N cleavage between fragment-XY and -XN.

In order to examine the possible contribution of the B-knob:b-hole interaction (B:b interaction) [18], reptilase-catalyzed fibrin polymerization was carried out as shown in Fig. 2. Polymerization in fragment-XY was more delayed and the value of OD was lowered than the case of thrombin, but massive fibrin network was formed. In contrast, polymerization in fragment-XN did not proceed at all. Protofibril formation occurred, but its growth was very slow and the lateral aggregation did not occur as ascertained by light scattering measurements. Because the B:b interaction does not work in these systems, these results are not explainable by the scheme that the decreased amount of FpB and lowered B:b interaction result in the delayed or no occurrence of lateral aggregation. Although the B:b interaction is not involved in the reptilase system, lateral aggregation is impaired even by the partial deletion of B β N region. As has been reported that the B:b interaction per se has no influence on the fibrin polymerization [9], B β N region must have important roles other than the B:b interaction, and B β N region might be essential for the normal fibrin polymerization.

Effect of mixing of intact fibrinogen on the polymerization of fragment-XY and -XN was examined. Mixing of intact fibrinogen by 5% (molar ratio) was carried out as illustrated in Fig. 3. Because the polymerization behavior of fragment-XY with thrombin was very similar to that of intact one, reptilase-catalyzed polymerization was examined. However, thrombin-catalyzed polymerization was carried out for fragment-XN. In order to clarify the process of protofibril growth, scattered light intensity is shown.

Polymerization of the mixture of fragment-XY with 5% intact fibrinogen was much more accelerated compared with that of fragment-XY alone, although the slope in the region of gentle increase of scattered light intensity, which corresponds to the process of protofibril growth, do not differ notably from that of fragment-XY. Because almost half of the B β N region is remained in fragment-XY, the addition of unimpaired B β N region of intact fibrinogen must not originate such a remarkable acceleration of polymerization, especially in the case without the B:b interaction. In other words, the observed acceleration could be resulted from the action of the α C region of intact one.

Thrombin-catalyzed polymerization of the mixture of fragment-XN with 5% intact fibrinogen, Fig. 3 (B), was drastically different from that of fragment-XN alone and Fig. 3 (A). Scattered light intensity of fragment-XN alone increased very gradually even up to 300 min,

Fig. 2. Reptilase-catalyzed fibrin polymerization of intact fibrinogen, fragment XY and fragment XN determined by the turbidity at 350 nm with the elapsed time. Polymerization in fragment-XY was delayed so much, and that in fragment-XN did not result in a gel.

Fig. 3. Reptilase-catalyzed polymerization of fragment-XY (A) and thrombin-catalyzed polymerization of fragment-XN (B) demonstrated as the scattered light intensity as a function of elapsed time together with 5% mixture of intact fibrinogen. Distinctive stepwise increase of intensity was observed for the mixture in (B).

protofibril formation proceeded very slowly, but any rapid increase of it, the characteristic signal of gelation and network growth, was not observed. On the contrary, results of the mixture showed a distinct stepwise increase of the scattered light intensity. Formation of

network was recognized, although the network was very fragile. Because the network formation was not observed in the 2% mixture of intact fibrinogen, it is probable that some threshold of normal fibrinogen content should exist for the network formation. The results of Fig. 3 (B) are consistent with Fig. 3 (A) with regard to the significance of B β N region as well as α C region. Fragile network formation might be due to B β N region of the intact fibrinogen, and therefore, it is suggested that B β N region functions substantially in the fibrin polymerization process.

Congenitally dysfunctional fibrinogen New York 1, in which B β 9-72 is deleted, is nonclottable and leads finally to demise [13]. The origins of this dysfibrinogenemia has been attributed to the conformational change: the deletion of B β 9-72 results in a breakage of disulfide bonding between B β 65Cys and A α 36Cys in the normal fibrinogen and then causes the conformational change in the central E region as well as A α N region. Additionally, since the region contains the β -wall to which thrombin binds [17], FpA release is likely to be impaired. Besides, the deletion of B β 9-72 could affect the function of α C domain, because FpA is involved in the interaction with α C domain together with B β N region [11]. Thus, the deletion of B β 9-72 results in not only the impaired function of B β N region, but also affects the release of α C-domain.

However, since des-B β 1-42 fibrinogen, B β 1-42 of which is cleaved, forms fibrin gel [14], the deletion of B β 1-42 in itself does not necessarily result in the defect of network formation and FpA release occurred. However, fibrin polymerization in des-B β 1-42 fibrinogen is so much delayed and weakened compared to the intact one. The cleavage of B β 1-42 causes not only the deletion of FpB essential for the B:b interactions, but also the defective thrombin binding. In addition to these, the existence of weak transient interactions (binding) of B β N region with the D region as well as α C-domain has been ascertained [15]. The impaired interactions of B β N region with D region might affect significantly to form the normal lateral aggregation necessary for the ordered fibrin fiber structure. In the meanwhile, it has been shown that formation of laterally oligomeric aggregation occurs in des-B β 1-42 fibrinogen through the α C- α C interaction predominantly to the protofibril growth using AFM technology [19].

Considered together with the present results of fragment-X, the contribution of α C region to the fibrin polymerization should not be ignored. In the recombinant variant fibrinogen, in which the binding of A-knob to a-hole is inhibited, the B:b interactions result in the delayed fibrin polymerization, and reptilase could not result in a fibrin gel [20]. When b-holes are blocked further by B-knob analog peptide GHRPam, thrombin-catalyzed polymerization was destructively impaired. Interaction of β N region with D region could be affected by the conformational change in the D region due to the binding of GHRPam [21]. Therefore, B β N alone is not attributable to the sufficient substance for the lateral aggregation, and it is suggested that B β N region might function cooperatively with α C-domain to form normal network in the fibrin polymerization.

In conclusion, polymerization behaviors of two types of fragment-X with thorough cleavage of α C region and differed magnitudes of B β N cleavage were examined focusing on the role of α C and B β N regions. Polymerization, especially lateral aggregation, depends on the cleavage of B β N region significantly. It is suggested that B β N regions might involve in the fibrin polymerization process cooperatively with α C-domains through the weak transient interaction with D region.

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