The binding of the anticoagulation factor Ⅰ from the venom of *Agkistrodon acutus* to activated factor Ⅹ

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Abstract Anticoagulation factor Ⅰ (ACF Ⅰ) from the venom of *Agkistrodon acutus* prolonged plasma prothrombin time (PPT) with dose-dependent manner and exhibited marked anticoagulant activity only at the concentration higher than its critical concentration (12 nmol/L). It was discovered that ACF Ⅰ formed a 1:1 complex with activated coagulation factor (FⅩa) in the presence of Ca^{2+} ions by the method of polyacrylamide gel electrophoresis. Both native ACF Ⅰ and decalcified ACF Ⅰ failed to form complexes with FⅩa in the absence of Ca^{2+}. Sr^{2+} ions were able to replace Ca^{2+} ions in the binding of ACF Ⅰ to FⅩa, but both Ba^{2+} ions and Tb^{3+} ions were ineffective. ACF Ⅰ was a new member of the Ⅸ/Ⅹ-bp family in the C-type lectin superfamily, and had a amino acid composition similar to the other members of this family. It was composed of 251 amino acid residues with a molecular weight of 29 603.6 u on non-reducing condition, determined by MALDI-TOF-MS, and a molecular weight of 14.7 ku on reducing condition, determined by the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Keywords: binding protein, anticoagulation factor, activated coagulant factor X, calcium ion.

Snake venoms contain various biologically active proteins which affect coagulation and platelet aggregation systems. An anticoagulation protein has been isolated from the venom of the habu snake (*Trimeresurus flavoviridis*) and designated as habu Ⅸ/Ⅹ-bp[1], since this protein forms a 1:1 complex with factor Ⅸ or Ⅹ in the presence of Ca^{2+} ions and prolongs the clotting time. Similar molecules have also been purified from *Trimeresurus flavoviridis* (i.e. habu Ⅸ-bp)[2], *Bothrops jararaca* (i.e. jararaca Ⅸ/Ⅹ-bp)[3] and *Echis carinatus leucogaster* (i.e. ECLV Ⅸ/Ⅹ-bp)[4]. Together these proteins form a unique subfamily, coagulation factor Ⅸ/factor X-binding protein family, in the C-type lectin superfamily[5].

Lu Yang and associates[6] purified an anticoagulation factor (ACF) from the venom of five-pace snake (*Agkistrodon acutus*) from southern Anhui Province, China. Our previous work showed that ACF was a Ca^{2+}-binding protein and had two Ca^{2+}-binding sites with different affinities. Calcium ions in ACF were essential for its anticoagulant activity and steric structure[7−9]. Recently, we isolated a novel anticoagulant protein designated as anticoagulation factor Ⅱ (ACF Ⅱ) from the venom of *Agkistrodon acutus*. For this reason, the anticoagulant protein (ACF) purified by Lu Yang is renamed anticoagulation factor Ⅰ (ACF Ⅰ). Both ACF Ⅰ and ACF Ⅱ possess marked anticoagulant activity, and are devoid of hemorrhagic and lethal activities, which may be useful as effective venom-based anticoagulants.

Presently, in order to understand the structure and function of the active site in ACF Ⅰ, and modify or engineer it to be an anticoagulant protein with strong anticoagulant activity and little side effect, we studied its mechanism of anticoagulation, and found that ACF Ⅰ formed a 1:1 complex with activated factor Ⅹ (FⅩa) in the presence of Ca^{2+} ions by a simple method of PAGE. We also investigated the dependence of binding of ACF Ⅰ to FXa on polyvalent cations other than Ca^{2+}, the molecular weight and amino acid composition of ACF Ⅰ, and compared it with other members of the Ⅸ/Ⅹ-bp family.

1 Materials and methods

(ⅰ) Materials. The lyophilized venom powder was obtained from the Tunxi Snakebite Institute (Anhui, China). ACF Ⅰ was purified by the method described previously[7]. FⅩa was a generous gift from Zhao Chao, the Research Center for Eco-Environmental Sciences, the Chinese Academy of

Sciences.

(ii) Determination of anticoagulant activity. It was performed through measuring plasma prothrombin time by the modified method of Cheng[10]. One tenth mL of the rabbit plasma was mixed with 0.1 mL test solution, 0.1 mL rabbit brain thromboplastin solution (25 g/L) and 0.8 mL physiological salt solution, and incubated for 1 min at 37℃. Then, 0.1 mL $CaCl_2$ (0.025 mol/L) was added and the clotting time was recorded from the point of addition of $CaCl_2$.

(iii) Analysis of the binding of ACF Ⅰ to FⅩa. PAGE was used for analysis of the binding of ACF Ⅰ to FXa. It was performed in 2.5% stacking gel at pH 6.7 and 7.5% separation gel at pH 8.9. Tris-Gly solution (pH 8.3) was used as the electrolyte buffer. Both sample solution as well as electrolyte buffer, and separation gel as well as stacking gel contained 1 mmol/L Ca^{2+} or other cations in order to keep ACF Ⅰ in the presence of Ca^{2+} or other cations in the electrophoresis process.

(iv) Determination of molecular weight. The accurate molecular mass of native ACF Ⅰ and the molecular weight of ACF Ⅰ reduced with β-mercaptoethanol were determined by LDI1700-MALDI-TOF-MS (Linear Scientific Inc., USA) and the conventional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), respectively.

(v) Amino acid analysis. The sample was hydrolyzed with 5.7 mol/L HCl for 24 h at 110℃, and then determined with a Waters 600E HPLC (Waters, USA). The tryptophan content of ACF Ⅰ was determined according to the NBS modification method of Spande[11].

(vi) Determination of concentrations of proteins. Concentrations of proteins were calculated from absorption coefficients ($A_{280}^{1\%}$) and relative molecular weights (M_r) using the following respective values: ACF Ⅰ, 29 603.6 and 30; and FⅩa, 45 300 and 10.

2 Results

(i) Dose-dependence of the anticoagulant activity of ACF Ⅰ and the effect of FⅩa on the anticoagulant activity of ACF Ⅰ. The relationship curve of plasma prothrombin time (PPT) to the concentration of ACF Ⅰ, as shown in fig. 1 (curve 1), indicates that ACF Ⅰ prolonged the PPT with dose-dependent manner. The PPT was not affected by ACF Ⅰ at the low concentration of ACF Ⅰ. When the concentration of ACF Ⅰ was higher than the critical concentration (12 nmol/L), the PPT increased rapidly with the increase of the concentration of ACF Ⅰ. It was reported that the content of coagulation factor Ⅹ (FⅩ) in plasma was 6—8 mg/L[12]. After all of FⅩ were activated, the concentration of FⅩa in the reaction mixture was 9—12 nmol/L. Interestingly, the value was near the critical concentration of ACF Ⅰ. Addition of 5 nmol/L FⅩa to the reaction mixture resulted in 6 nmol/L right-shift of the dose-dependent curve (fig. 1, curve 2), which has a very similar shape to curve 1. More interestingly, the critical concentration of ACF Ⅰ increased by 6 nmol/L correspondingly, which was also near the concentration of the added FⅩa. From these results we speculated that ACF Ⅰ prolonged the clotting time probably by binding to FⅩa.

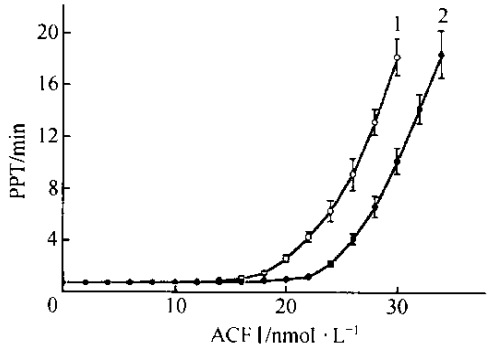

Fig. 1. Dose-dependent prolongation of PPT by the purified ACF Ⅰ. 1, Not containing added FⅩa; 2, containing 5 nmol/L added FⅩa.

(ii) PAGE analysis of the binding of ACF Ⅰ to FⅩa. If ACF Ⅰ binds with FⅩa, it may migrate on PAGE at a different rate due to the changes of the molecular weight and net charge, thus we used PAGE to analyze the binding of ACF Ⅰ to FⅩa. As shown in fig. 2, in the absence of Ca^{2+}, the mixture of ACF Ⅰ and FⅩa (molar ratio, 1:1) produced two bands (fig. 2(a), lane 2) on the PAGE, corresponding to the band of ACF Ⅰ (fig. 2(a), lane 3) and the band of FⅩa (fig. 2(a), lane 1)

respectively. Obviously, ACF Ⅰ did not form a complex with FⅩa in the absence of Ca^{2+}. However, in the presence of 1 mmol/L Ca^{2+}, the mixture of ACF Ⅰ and FⅩa (molar ratio, 1∶1) produced a new band (fig. 2(b), lane 1), corresponding neither to the band of ACF Ⅰ (fig. 2(b), lane 4), nor to the band of FⅩa (fig. 2(b), lane 2). This result indicated that ACF Ⅰ formed a complex with FⅩa in the presence of 1 mmol/L Ca^{2+}. In the same concentration of Ca^{2+}, the mixture of ACF Ⅰ and FⅩa (molar ratio, 1.5:1) produced two bands (fig. 2(b), lane 3), corresponding to the complex of FⅩa and ACF Ⅰ (fig. 2(b), lane 1) and ACF Ⅰ (fig. 2(b), lane 4) respectively. The results showed that ACF Ⅰ formed a 1:1 complex with FXa in the presence of 1 mmol/L Ca^{2+}.

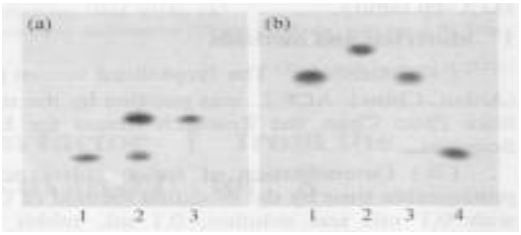

Fig. 2. Polyacrylamide gel electrophoresis. (a) In the absence of Ca^{2+}: 1, 10 μL FⅩa; 2, 10 μL ACF Ⅰ + 10 μL FⅩa; 3, 10 μL ACF Ⅰ. (b) In the presence of 1 mmol/L Ca^{2+}: 1, 10 μL ACF Ⅰ + 10 μL FⅩa; 2, 10 μL FⅩa; 3, 15 μL ACF Ⅰ + 10 μL FXa; 4, 10 μL AFThe concentrations of both ACF Ⅰ and FⅩa are 30 μmol/L.

The effects of polyvalent cations other than Ca^{2+} on the binding of ACF Ⅰ to FⅩa were also investigated by PAGE. Binding to FⅩa had not absolute requirement for Ca^{2+} ions. Sr^{2+} ions could be substituted for Ca^{2+} ions in binding of ACF Ⅰ to FⅩa. However, both Ba^{2+} and Tb^{3+} ions were ineffective. ACF Ⅰ could not bind to FⅩa in the presence of 1 mmol/L EDTA, too.

(ⅲ) Determination of molecular weight of ACF Ⅰ. In comparison with traditional methods of determining molecular weight, for example, HPLC and SDS-PAGE, MALDI-TOF-MS has the advantages of higher accuracy, higher resolution, higher sensitivity, lower sample amount and shorter analysis time. As shown in fig. 3, the accurate molecular weight of ACF Ⅰ determined by MALDI-TOF-MS was 29 603.6 u (peak 2). Peaks 1, 3 and 4 represent the ions of ACF Ⅰ and its polymer in different charge states $[M+2H]^{2+}$, $[3M+2H]^{2+}$ and $[2M+H]^{+}$, respectively. By SDS-PAGE measurement, ACF Ⅰ gave a single band corresponding to about 14.7 ku, on reducing condition.

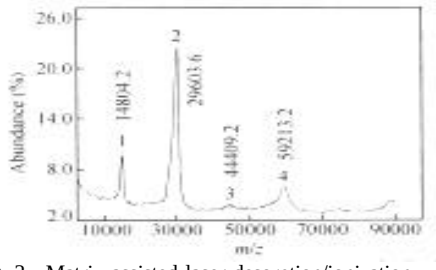

Fig. 3. Matrix assisted laser desorption/ionization time of flight mass spectrum of ACF Ⅰ.

Table 1 Amino acid composition of ACF Ⅰ

Amino acid	ACF Ⅰ	Habu ⅨⅩ-bp[1]	ECLV ⅨⅩ-bp[4]	Habu Ⅸ-bp[2]
Asx	20.1 (20)	23	18	22
Ser	22.8 (23)	25	27	26
Glx	34.1 (34)	37	35	37
Gly	14.4 (14)	14	18	16
His	8.3 (8)	6	7	6
Arg	3.6 (4)	8	6	7
Thr	12.2 (12)	8	12	9
Ala	15.4 (15)	20	12	19
Pro	4.3 (4)	4	6	6
1/2Cys	13.5 (14)[a]	14	14	14
Tyr	13.6 (14)	11	9	11
Val	11.8 (12)	15	13	14
Met	2.8 (3)	5	1	4
Lys	17.7 (18)	16	29	16
Ile	9.3 (9)	6	6	6
Leu	10.2 (10)	11	13	10
Phe	23.4 (23)	15	13	15
Trp	14[b]	14	17	14
Total	251	252	256	252

a) Determined as cysteic acid after performic acid oxidation. b) Residue values obtained by the NBS modification method of Spande[11].

(iv) Amino acid analysis. The amino acid composition of ACF I is given in table 1. ACF I contained 251 amino acid residues and was rich in glutamic acid, aspartic acid, serine, phenylalanine, glutamine and asparagine: together they constituted 40.6% of the total amino acid residues. The high content of tryptophan (14 residues per molecule) contributed a lot to the high absorption coefficient of ACF I at 280 nm, i.e. $A_{280}^{1\%} = 30$.

3 Discussion

The anticoagulant proteins from snake venoms are classified into two categories: anticoagulant enzymes and non-enzymatic anticoagulants. The anticoagulant enzymes, such as phospholipases, fibrinogenolytic enzymes, protein C activators and proteolytic enzymes, convert coagulation factors into degraded inactive forms. The non-enzymatic anticoagulants inhibit the activation of prothrombin[13]. The coagulation factor IX/factor X-binding proteins are non-enzymatic anticoagulants. These anticoagulant proteins form 1:1 complexes with either coagulation factor IX (FIX), activated coagulation factor IX (FIXa) or factor X (FX), activated factor X (FXa) in a calcium-dependent fashion, and thereby block the amplification of the coagulation cascade. ACF I is devoid of any enzymatic activities[1)], and forms a 1:1 complex with FXa in the presence of Ca^{2+}. Therefore, it is a new member of the IX/X-bp family.

We have observed that ACF I prolongs plasma prothrombin time in dose-dependent manner and shows marked anticoagulant activity only at the concentration higher than its critical concentration. When the concentration of ACF I is lower than its critical concentration, part of FXa in the reaction mixture bind to ACF I, and the free FXa, with other coagulation factors, can still activate prothrombin to be thrombin which causes fibrinogen to clot; therefore, PPT does not change by the addition of ACF I. Because the concentration of FXa in the reaction mixture is near the critical concentration of ACF I, when the concentration of ACF I is higher than the critical concentration, most of FXa in the reaction mixture have bound to ACF I, and the free FXa is so little that the reaction of activating prothrombin by the FXa-FVa-Ca^{2+}-phospholipid complex becomes the rate-limiting step. As a result, PPT increases rapidly with the increase of the concentration of ACF I. After addition of 5 nmol/L FXa to the reaction mixture, the concentration of ACF I must be increased equally in order to make most of FXa bind to ACF I, as a result, the critical concentration of ACF I increases by 6 nmol/L correspondingly.

The molecular weights of two chains of ACF I are about 14.7 ku determined by SDS-PAGE. These results agree with the molecular weight of 29 603.6 u measured with MALDI-TOF-MS. Similar to ACF I, the molecular weight of habu IX/X-bp is 29 400 u and the molecular weights of its two chains are 14 830 and 14 440 u respectively. The amino acid composition of ACF I is similar to habu IX/X-bp, ECLV IX/X-bp and habu IX-bp (table 1). All contain relatively large amounts of acidic amino acids (i.e. glutamic acid and aspartic acid), serine and tryptophan. Both habu IX/X-bp and ECLV IX/X-bp contain 14 half-cystines and have the same disulfide-bonding pattern of seven disulfides. ACF I also contains 14 half-cystines which may result in the formation of seven disulfide bridges within the molecule like habu IX/X-bp and ECLV IX/X-bp.

It was reported that the binding of jararaca IX/X-bp to FX had an absolute requirement for Ca^{2+} ions[3]. Sr^{2+}, Mn^{2+} and Mg^{2+} ions could be substituted for Ca^{2+} ions in the binding of jararaca IX/X-bp to FIX, but Ba^{2+}, Zn^{2+} and La^{3+} ions were ineffective in all cases. Sr^{2+} ions were able to substitute Ca^{2+} ions in the binding of ECLV IX/X-bp to FIX[4]. However, Sr^{2+} ions were not able to replace Ca^{2+} ions in the binding of ECLV IX/X-bp to FX. Mg^{2+}, Ba^{2+}, Zn^{2+}, Cu^{2+} and Mn^{2+} ions could not substitute Ca^{2+} ions for inducing FIX or FX to bind to ECLV IX/X-bp. The results of PAGE show that Sr^{2+} ions

1) Xu, X. L., Liu, Q. L., Xie, Y. S. et al., Purification and characterization of anticoagulation factors from the venom of *Agkistrodon acutus*, Toxicon, in press.

could substitute Ca^{2+} ions in the binding of ACF Ⅰ to FⅩa; however, both Ba^{2+} and Tb^{3+} ions were ineffective.

Can ACF Ⅰ bind to FⅩ, FⅨ or FⅨa like other members of this family? Do Ca^{2+} ions act as "bridges" between the two proteins, or alter the conformation of ACF Ⅰ to make "a lock and key"? Further studies on these issues are useful in the efforts to elucidate the mechanisms of anticoagulation of snake proteins, and structure-function relationships of proteins.

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