Interaction of Bothrojaracin with Prothrombin

Russolina B. Zingali M. Lucia Bianconi Robson Q. Monteiro

Departamento de Bioquímica Médica, ICB/CCS, Universidade Federal do Rio de Janeiro, Brazil

Key Words

Bothrojaracin · Prothrombin · Proexosite I · Factor Va · Prothrombinase

Abstract

Bothrojaracin (BJC) is a 27-kD protein from *Bothrops jararaca* venom that interacts with α -thrombin ($K_D = 0.7$ nM) through both anion-binding exosites I and II. Recently, it has been shown that BJC interacts with the exosite I precursor (proexosite I) on human prothrombin ($K_D = 75$ nM), forming a 1:1 Ca^{2+} -independent noncovalent complex with the zymogen. Complex formation was associated with inhibition of zymogen activation by *Oxyuranus scutellatus* venom. In addition, BJC strongly decreased the prothrombin activation by factor Xa only in the presence of factor Va. A similar effect was observed in the presence of phospholipids, suggesting that BJC specifically inhibits the interaction of prothrombin with factor Va. It is proposed that BJC has two independent mechanisms

for anticoagulation: (1) inhibition of exosite-I-dependent activities on α -thrombin, and (2) inhibition of prothrombin activation through interaction with proexosite I.

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Introduction

Thrombin is formed by the cleavage of two peptide bonds in prothrombin by blood coagulation factor Xa, with the release of prothrombin fragments 1 and 2. These reactions are modulated by calcium, membranes containing acidic phospholipids, and the protein cofactor, factor Va, which assemble with factor Xa and prothrombin to form the prothrombinase complex [1]. It is generally observed that phospholipids increase the substrate affinity while factor Va enhances the catalytic rate of the reaction [2].

During prothrombin activation, besides the exposure of the catalytic site, additional regulatory domains become available [3–4].

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Dr. Russolina B. Zingali
Departamento de Bioquímica Médica/ICB/CCS
Bloco H – 2º Andar – Ilha do Fundão
Rio de Janeiro, RJ 21941-590 (Brazil), Tel. +55 21 2562 6782
Fax +55 21 2270 8647, E-Mail lzingali@bioqmed.ufrj.br

Anion-binding exosite I is a positively charged region responsible for the high specificity of the enzyme towards macromolecular substrates, cofactors and some inhibitors [5, 6]. Exosite II is referred to as heparin binding site [7]. Recently, it has been shown that exosite I is partially exposed on prothrombin in a precursor state (proexosite I) and is fully exposed on thrombin [8].

Binding of prothrombin to factor Va is believed to involve fragment 2 domain of prothrombin [9–11]. More recently, it was shown that C-terminal hirudin peptides inhibit prothrombin activation by factor Xa only in the presence of factor Va [12]. This observation suggested the involvement of proexosite I in substrate interactions with the cofactor.

Herein we show that the interaction of bothrojaracin (BJC) with prothrombin occurs through the proexosite I, and also that the formation of this complex impairs prothrombin activation in the presence of factor Va.

Interaction of BJC with Thrombin

BJC was isolated from the venom of the Brazilian snake jararaca (*Bothrops jararaca*) [13]. It is a disulfide-linked heterodimer that belongs to the C-type (Ca^{2+} -dependent) lectin-like related protein family, which contains structurally homologous proteins displaying distinct biological functions. BJC has been characterized as a potent and selective thrombin inhibitor ($K_D = 0.7 \text{ nM}$) since it binds to both exosites I and II [14], forming a 1:1 non-covalent complex. Binding of BJC to thrombin modulates, but does not impair the cleavage of tripeptide chromogenic substrates [16]. On the other hand, complex formation results following inhibition of exosite-I-related functions, such as fibrinogen clotting, platelet aggregation, thrombomodulin-dependent protein C activation and factor V activation [13,

15]. In addition, exosite II-related functions are also impaired, as demonstrated by blockage of heparin-dependent antithrombin inactivation of the thrombin-BJC complex [14].

Interaction of BJC with Prothrombin

BJC forms a calcium-independent, 1:1 complex with human prothrombin [14, 17]. Nevertheless, no interaction was observed with prothrombin fragment 1, which contains the Gla and first kringle domains, or fragment 2, that is constituted by the second kringle domain. Thus these data suggest that the site of interaction with BJC is located in the catalytic domain of prothrombin. Isothermal titration calorimetry (ITC) showed that the binding of prothrombin to BJC is endothermic ($\Delta H = 13.5 \pm 1.8 \text{ kcal/mol}$), indicating that the binding is entropically driven, with free energy $\Delta G = -9.7 \text{ kcal/mol}$, and entropy term $-T\Delta S = -3.8 \text{ kcal/mol}$ [17].

Anderson et al. [8] recently characterized the partially exposed exosite I (proexosite I) on prothrombin. Binding experiments showed that BJC and sulfated C-terminal hirudin compete for the same site. In fact, BJC presents the highest affinity for proexosite I described to date (table 1). Interestingly, BJC and C-terminal hirudin peptides display a similar ~ 100 -fold increase in affinity for exosite I when prothrombin is activated to thrombin (table 1). These observations strongly suggest that proexosite I is a low-affinity, precursor state of exosite I.

Effect of BJC on Prothrombin Activation

It has previously been shown that BJC inhibits factor V activation by thrombin [15]. Therefore the thrombin formation by the pro-

Fig. 1. Anticoagulant X anti-prothrombin-converting activity of BJC. Prothrombin (100 nM) activation was carried out in 50 mM Tris-HCl, 150 mM NaCl, 5 mM CaCl₂, 1 mg/ml PEG 8000, pH 7.5 containing varying amounts of BJC and 0.2 mM S-2238 (▲) or 2 mg/ml human fibrinogen (●). The reaction was started by addition of 10 µg/ml *O. scutellatus* venom. The experiments performed in the absence of BJC were taken as 100%. Data represent mean ± SD of two independent determinations.

Table 1. Proexosite I ligands and their affinities for prothrombin and thrombin binding

Ligand	K_D , μM prothrombin/thrombin	Ratio	Reference
Hirudin ⁵⁴⁻⁶⁵	45/0.65	69	8
Hirudin ⁵⁴⁻⁶⁵ (SO ³⁻)	3.2/0.025	128	8
BJC	0.076/0.00062	126	17, 14

thrombinase complex in the presence of factor V is decreased [15]. Since BJC also interacts with prothrombin it was reasonable to speculate that this protein could interfere with prothrombin activation. Several prothrombin activators isolated from snake venoms are currently known [18]. Scuterin is a 300-kD enzyme from *Oxyuranus scutellatus* venom that is structurally related to a factor Xa-factor Va complex [19]. Formation of the BJC-prothrombin complex was associated with

partial inhibition of zymogen activation by *O. scutellatus* venom (fig. 1 and 2) [20], thus indicating that BJC can also block prothrombin activation. In addition, thrombin formed in the assay showed a progressive decrease in the fibrinogen-clotting activity. Thus, at ~1 µM BJC, prothrombin activation by *O. scutellatus* was decreased by ~50% while the thrombin formed was completely inactive towards fibrinogen (fig. 1). In a purified system, BJC decreased prothrombin activation by factor

Fig. 2. Effect of BJC on prothrombin activation. Prothrombin (500 nM) activation was carried out in 50 mM Tris-HCl, 150 mM NaCl, 5 mM CaCl₂, 1 mg/ml PEG 8000, pH 7.5 containing 1 mg/ml bovine serum albumin. Reaction was started by addition of 10 µg/ml *O. scutellatus* venom, or 10 nM factor Xa, or 10 nM factor Xa/1 nM factor Va, or 10 nM factor Xa/

10 µM phospholipid vesicles (PCPS; 75% phosphatidylcholine, 25% phosphatidylserine), or 100 pM factor Xa/10 pM factor Va/10 µM phospholipid vesicles. Experiments were performed in the absence (taken as 100% activation) or in the presence of 1.5 µM BJC. Data represent mean ± SD of three independent determinations.

Xa in the presence of factor Va, with 70% inhibition. However, no effect was observed in the absence of the cofactor (fig. 2). This result agrees with previous studies in which C-terminal hirudin peptides also inhibited factor-Va-accelerated prothrombin activation [12]. We suggested that the effect of BJC is specific towards prothrombin interaction with factor Va. Experiments performed in the presence of model membranes (75% phosphatidylcholine, 25% phosphatidylserine) showed a similar profile although the inhibitory effect of BJC was significantly lower than that observed in the absence of phospholipids (fig. 2). Thus, BJC caused a 35% inhibition of prothrombin activation by factor Xa in the presence, but not in the absence of factor Va. Altogether these data reinforce the hypothesis that proexosite I participates in the interaction of prothrombin with factor Va.

BJC as Anticoagulant

We have previously shown that BJC specifically interacts with prothrombin in human plasma [20]. As expected, BJC is an effective inhibitor of plasma clotting [15, 20], which is also able to reduce thrombin formation after activation of the intrinsic pathway of blood coagulation [20]. Studies of BJC interaction with either thrombin or prothrombin suggest that, in vivo, BJC produces an anticoagulant effect. Two distinct mechanisms may thus be involved (fig. 3): (1) BJC binds to the generated thrombin, and inhibits the exosite-1-dependent activities such as fibrinogen clotting, factor V activation, platelet activation and possibly factor VIII activation. It is unclear whether BJC inhibits factor XI and/or factor XIII activation, and (2) whether BJC interacts with prothrombin and interferes

Fig. 3. Anticoagulant mechanism of BJC. Mechanism 1 is based on the inhibition of exosite-I-related activities. Mechanism 2 is based on the inhibition of prothrombin activation through interaction with proexosite I.

with proexosite-I-mediated interactions with factor Va, thus inhibiting the zymogen activation.

Future Perspectives

The physiological role of proexosite I in prothrombin function/activation remains unclear. The specific interference of BJC in fac-

tor-Va-accelerated prothrombin activation suggests that this region might modulate the interaction of the cofactor with the zymogen in vivo during its activation. At this point, proexosite I may be a potential target for the development of new antithrombotic drugs. Furthermore, the higher binding affinity of BJC when compared to Hir⁵⁴⁻⁶⁵(SO³⁻) point to this molecule as a tool for future studies on that newly characterized prothrombin site.

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