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Research paper

VaH3, one of the principal hemorrhagins in *Vipera ammodytes ammodytes* venom, is a homodimeric P-IIIc metalloproteinase

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ABSTRACT

Hemorrhage is the most potent manifestation of envenomation by *Vipera ammodytes ammodytes* (*V. a. ammodytes*) venom in man. A detailed description of the venom components contributing to this effect is thus medically very important. We have characterized a novel component, termed here VaH3, as a potently hemorrhagic snake venom metalloproteinase (SVMP). Its proteolytic activity and overall stability depend on the presence of Zn^{2+} and Ca^{2+} ions. The molecular mass of this slightly acidic molecule, determined by MALDI/TOF analysis, is 104 kDa. Chemical reduction and S-carbamoylmethylation result in a single monomer of 53.7 kDa. *N*-deglycosylation decreased this mass by 4.6 kDa. The complete amino acid sequence of VaH3 was determined by protein and cDNA sequencing, showing that each of the identical glycoprotein subunits comprise a metalloproteinase, a disintegrin-like domain and a cysteine-rich domain, VaH3 belongs to the P-IIIc class of SVMPs. It shows strong sequence similarity to vascular endothelial cell apoptosis-inducing reprolysins. Anti-ammodytagin antibodies strongly cross-reacted with VaH3 and completely neutralized its hemorrhagic activity in rat, despite the fact that the two hemorrhagic P-III SVMPs from *V. a. ammodytes* venom do not share a very high degree of amino acid sequence identity. In spite of its narrow proteolytic specificity, VaH3 rapidly cleaved some basal membrane and extracellular matrix proteins, such as collagen IV, fibronectin and nidogen. Moreover, it also hydrolyzed plasma proteins involved in blood coagulation. It is an effective α-fibrinogenase that cleaves prothrombin and factor X without activating them. The degradation of these proteins likely contributes to the hemorrhagic activity of VaH3. A three-dimensional model of VaH3 was built to help explain structure–function relationships in ADAM/ADAMTS, a family of proteins having significant therapeutic potential and substantial sequence similarity to VaH3.

1. Introduction

Zn^{2+}-dependent metalloproteinases play a major part in the pathological effects of viperid snake bites, the most pronounced being local and systemic hemorrhage, local tissue damage and coagulopathy [1,2]. Snake venom metalloproteinases (SVMPs) are therefore important targets in antivenom therapy. SVMPs have also found other applications beneficial for human health. For example, they have been used in diagnosis of some clotting disorders, and employed as template molecules for the development of novel thrombolytic drugs [3] and for structure–function studies of the respective mammalian orthologues.

Abbreviations: ADAM, a disintegrin and metalloproteinase; ADAMTS, ADAM with thrombospondin type-1 motif; BM, basal membrane; CR, cysteine-rich; Dis, disintegrin-like; DSF, differential scanning fluorimetry; ECM, extracellular matrix; EC, endothelial cell; FX, factor X; GPIb, platelet glycoprotein Ib; MEROPS, M12B clan of zinc metalloproteinases; MP, metalloproteinase; PPP, platelet-poor plasma; PRP, platelet-rich plasma; RMSD, root-mean-square deviation; SVMP, snake venom metalloproteinase; *V. a. ammodytes*, *Vipera ammodytes ammodytes*; VaH3, *Vipera ammodytes* hemorrhagin 3; vWF, von Willebrand factor.

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SVMPs are grouped into three major classes, P-I, P-II and P-III, according to their domain organization [4]. While P-I SVMPs are composed of a single catalytic metalloproteinase (MP) domain, P-II SVMPs have, in addition, a disintegrin domain, and P-III SVMPs a disintegrin-like (Dis) domain and a cysteine-rich (CR) domain. P-II and P-III SVMPs are divided further into several subclasses, depending on their post-translational proteolytic processing and dimerization [4]. Non-catalytic Dis and CR domains contain several substrate-binding sites located in the so-called disintegrin loop and in the hyper-variable region [5,6]. These binding sites define substrate specificity and localize P-III SVMPs at their site of action.

The extracellular matrix (ECM) ensures the mechanical stability of microvessels. Binding and subsequent hydrolysis of the key components of ECM by SVMPs are the central steps in their hemorrhagic action [7]. The evidence supports a two-step mechanism of SVMP-induced hemorrhage [8]. Proteolysis of the major components of basal membrane (BM), laminin, type IV collagen, nidogen and perlecan [9,10], and of proteins that play a key role in the mechanical stability of BM, *e.g.* non-fibrillar collagens [10,11], weakens the capillary wall. The hydrostatic pressure of the blood then distends and eventually disrupts the destabilized vessels, leading to extravasation of blood. The hemorrhagic effect of SVMPs is further potentiated by their ability to inhibit platelet aggregation and to hydrolyze plasma proteins involved in the process of blood clotting, such as different coagulation factors and von Willebrand factor (vWF) [3].

Phylogenetically, SVMPs are most closely related to the mammalian ADAM (A Disintegrin And Metalloproteinase) family of proteins. Together with ADAM and ADAM with a thrombospondin type-1 motif (ADAMTS) proteins, they belong to the adamalysin/reprolysin/ADAM family and the M12B clan of zinc metalloproteinases (MEROPS classification: http://merops.sanger.ac.uk/cgi-bin/famsum?family=M12B).

ADAM/ADAMTSs have an MP + Dis + CR (or MDC) domain architecture identical to that of P-III SVMPs [12]. While ADAMTS proteinases are secreted, ADAMs are membrane-bound sheddases that proteolytically process cell surface proteins involved in cell–ECM and cell–cell adhesion and signaling. As such, they possess significant therapeutic potential [13]. Since three-dimensional structures of only fragments of mammalian ADAM/ADAMTS proteins are available, structural analysis of homologous P-III SVMPs is also important in studies of structure–function relationships in ADAM/ADAMTS proteins [6,14–16].

Vipera ammodytes ammodytes is the most venomous snake in Europe. The most critical manifestations of its venomous bite are hemorrhage and local tissue damage [17]. These effects are induced mostly by SVMPs, three of which have already been characterized, VaH1, VaH2 [18] and ammodytagin [19], all three belonging to the P-III class SVMPs. The structurally similar but non-hemorrhagic fibrin(ogen)olytic P-IIIa SVMP, ammodytase [20], and two factor X activating P-IIId SVMPs, VAFXA-I and -II [21], also contribute to the interference of the venom with the hemostatic process.

Here we describe the isolation and characterization of another potently hemorrhagic *V. a. ammodytes* SVMP, possessing also high fibrinogenolytic activity, that was indicated in our previous study [18]. We named it VaH3 (***V****ipera* ***a****mmodytes* **H**emorrhagin **3**). Importantly, we report the first complete primary structure of a *V. a. ammodytes* SVMP. VaH3 is a homodimeric P-IIIc class SVMP. Structurally, it is very similar to vascular endothelial cell (EC) apoptosis-inducing reprolysins, suggesting that it also possesses this activity. Based on the crystal structure of one of these, *Crotalus atrox* (*C. atrox*) VAP1, a three-dimensional model of VaH3 was constructed. VaH3 is one of the key hemorrhagic toxins in *V. a. ammodytes* venom, and the neutralization of its pathological activity is very important for effective antivenom therapy of patients bitten by *V. a. ammodytes*. We show that antibodies raised against ammodytagin also completely neutralize VaH3 hemorrhagic activity.

2. Materials and methods

Crude *V. a. ammodytes* venom was obtained from the Institute of Immunology, Zagreb, Croatia. All other chemicals were of analytical or sequencing grade. All animal work was in accordance with Croatian Law on Animal Welfare (2006) which strictly complies with EC Directive 86/609/EC.

2.1. Isolation of VaH3

VaH3 was isolated using the method of Leonardi et al. [18,21]. Unbound fraction from anion-exchange chromatography on DEAE-Sephacel was applied to a hydroxyapatite-Ultrogel column (Serva, Germany) previously equilibrated with 10 mM Na_2HPO_4, 20 mM imidazole, pH 7.0 (buffer A). The unadsorbed fraction containing VaH3 was applied to a cation-exchange Mono-S HR 5/5 FPLC column (Amersham Biosciences, UK) equilibrated with 20 mM MES, pH 6.5, containing 2 mM $CaCl_2$. Proteins were eluted with a linear gradient of NaCl from 0 to 1 M in the equilibration buffer over 30 min at a flow rate of 1 mL/min.

2.2. SDS-polyacrylamide gel electrophoresis and isoelectric focusing

SDS-PAGE analysis of the isolated protein was performed on 12.5% (w/v) polyacrylamide gels under reducing and non-reducing conditions according to Laemmli [22]. Molecular mass standards used were from Fermentas (Lithuania). Isoelectric focusing (IEF) was performed using a PhastSystem (Amersham Pharmacia Biotech, Sweden), following the manufacturer's instructions. PhastGel 4–6.5 and 3–9 slabs (0.35 × 43 × 50 mm) and pI standards (3.5–9.3) were used. Proteins were visualized by staining with Coomassie Brilliant Blue R250.

2.3. N-deglycosylation

Vacuum-dried VaH3 samples (4 μg) were denatured with 1% (w/v) SDS at 100 °C for 5 min then mixed with 1.5% (w/v) CHAPS in 50 mM Na_2HPO_4, pH 7.5. Three U of peptide *N*-glycosidase F (PNGF, Roche, Germany) were added to half the sample; the other half served as control. Both were incubated at 37 °C overnight and subjected to SDS-PAGE on 12.5% gel under reducing conditions.

2.4. Differential scanning fluorimetry

Differential scanning fluorimetry (DSF) is a rapid screening method for evaluating the influence of buffer components on the stability of a protein [23]. The temperature at which a protein unfolds is measured by an increase in the fluorescence of a dye, in our case SYPRO Orange, with affinity for hydrophobic parts of the protein, which are exposed as the protein unfolds. For each screen, 10 μL of experimental mixture contained 1 μL of protein solution (final concentration ~0.01 mM), 2 μL of 50-fold water-diluted SYPRO Orange stock solution (Sigma, Germany) and 7 μL of the relevant buffer to final concentrations shown in Fig. 2. Fluorescence of SYPRO Orange was monitored in the range from 25 to 99 °C, increasing the temperature at 0.1 °C/min. Following the subtraction of the background fluorescence, the protein melting temperature (T_m) was calculated from the first derivative of the fluorescence curve using Prism 5.00 (GraphPad Software, USA).

2.5. Endoproteinase Lys-C digestion of VaH3 and purification of fragments

VaH3 was digested with endoproteinase Lys-C for 24 h at 37 °C in 20:1 (w/w) ratio in 100 μL of 50 mM Tris/HCl buffer, pH 8.7,