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Crystallization and preliminary X-ray analysis of coagulation factor IX-binding protein from habu snake venom at pH 6.5 and 4.6

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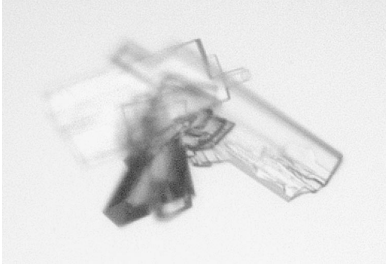


Coagulation factor IX-binding protein isolated from *Trimeresurus flavoviridis* (IX-bp) is a C-type lectin-like protein. It is an anticoagulant protein consisting of homologous subunits *A* and *B*. The subunits both contain a Ca^{2+}-binding site with differing affinity (K_d values of 14 and 130 μ*M* at pH 7.5). These binding characteristics are pH-dependent; under acidic conditions, the affinity of the low-affinity site was reduced considerably. In order to identify which site has high affinity and also to investigate the Ca^{2+}-releasing mechanism, IX-bp was crystallized at pH 6.5 and 4.6. The crystals at pH 6.5 and 4.6 diffracted to 1.72 and 2.29 Å resolution, respectively; the former crystals belong to the monoclinic space group $P2_1$, with unit-cell parameters a = 60.7, b = 63.5, c = 66.9 Å, β = 117.0°, while the latter belong to the monoclinic space group $C2$, with a = 134.1, b = 37.8, c = 55.8 Å, β = 110.4°.

1. Introduction

Snake venoms contain a wide variety of protein components affecting haemostasis. One such group consists of the C-type lectin-like proteins (CLPs; Atoda *et al.*, 1991). The name CLP was derived from the primary structure and disulfide-bond arrangement of the coagulation factor IX/X-binding protein (IX/X-bp) from the venom of *Trimeresurus flavoviridis* (habu snake; Atoda *et al.*, 1991; Atoda & Morita, 1993). IX/X-bp is a heterodimeric protein consisting of homologous subunits *A* (129 amino-acid residues) and *B* (123 amino-acid residues) linked by a disulfide bond. The amino-acid sequence of each subunit shows homology with the carbohydrate-recognition domains of the classic C-type lectins, although lectin activity has not been reported. We first determined the crystal structure of a CLP using IX/X-bp (Mizuno *et al.*, 1997). The structure showed that the two subunits were intimately associated by domain swapping and that this dimerization resulted in the disruption of the lectin active site and the creation of a concave surface serving as an expected coagulation factor binding site. To date, the crystal structures of factor IX binding protein (IX-bp) from habu snake venom (Mizuno *et al.*, 1999), the complex between IX-bp and the γ-carboxyglutamic acid (Gla) domain of bovine factor IX (Shikamoto *et al.*, 2003) and the complex between factor X binding protein (X-bp) from *Deinagkistrodon acutus* and the Gla domain of factor X (Mizuno *et al.*, 2001) have been determined. These CLP structures are very similar to each other and the complex structures have revealed that the binding site is in fact the concave surface.

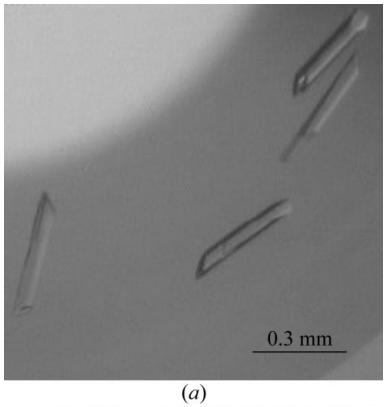
Another interesting feature of IX-bp, X-bp and IX/X-bp is that they possess Ca^{2+}-binding sites that are common to them but differ from those of classic C-type lectins. Each domain binds to a Ca^{2+} ion. The residues in IX-bp coordinating with Ca^{2+} ions are Ser41, Glu43, Glu47 and Glu128 in subunit *A* and Ser41, Gln43, Glu47 and Glu120 in subunit *B*. These residues are completely conserved in X-bp and IX/X-bp. In IX/X-bp, intrinsic fluorescence owing to tryptophan was reported to be elevated by the addition of Ca^{2+} ions to the dialyzed protein. This indicated that Ca^{2+} binding induces conformational changes to stabilize the protein structure. The Ca^{2+}-binding affinity at pH 7.5 differed by tenfold (25 and 200 μ*M*) between the two sites of IX/X-bp and the Ca^{2+}-binding kinetics for the fluorescence change suggested that only the higher affinity site contributed to the conformational change (Sekiya *et al.*, 1995). When the pH was

decreased to 6.5, the affinity of the high-affinity binding site was reduced only slightly, but that of the low-affinity site was reduced considerably (Sekiya *et al.*, 1995). In the case of IX-bp, the Ca^{2+}-binding affinities of the two binding sites were 14 and 130 μ*M* at pH 7.5 (Atoda *et al.*, 1995) and a similar pH-dependence of Ca^{2+}-binding characteristics is expected from the high similarity around the Ca^{2+}-binding sites in IX-bp and IX/X-bp. To make sure of this, we conducted a Ca^{2+}-binding assay by fluorescence measurement. We then prepared crystals of IX-bp from habu snake venom at pH 6.5 in the presence of a low concentration of Ca^{2+} in order to investigate which subunit contained the high-affinity Ca^{2+}-binding site. Furthermore, we prepared crystals of IX-bp at pH 4.6 as the apo form in order to study the pH-dependent Ca^{2+}-releasing mechanism by comparing the structures around the Ca^{2+}-binding sites at different pH values including pH 7.8 (Mizuno *et al.*, 1999).

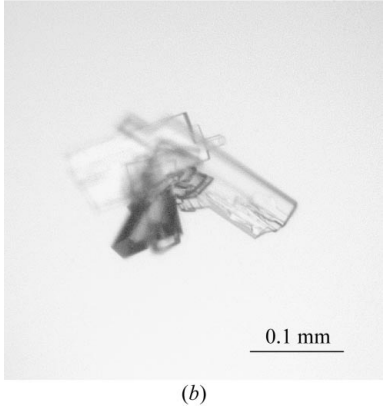
2. Materials and methods

2.1. Purification

Habu IX-bp (27.5 kDa) was purified from the lyophilized venom of *T. flavoviridis* (purchased from Japan Snake Institute, Gumma) as described previously (Atoda *et al.*, 1995). An additional purification by gel-filtration using Superdex 75pg (Amersham Biosciences) was carried out prior to crystallization. Protein solutions were then dialysed against buffer at either pH 6.5 (20 m*M* MES buffer) or 4.6 (20 m*M* acetate buffer). IX-bp at pH 4.6 was prepared by dialysis against Sigma chelating resin (1 g l^{-1}) containing acetate buffer (20 m*M*, pH 4.6).



(*a*)



(*b*)

Figure 1
Crystals of habu IX-bp at (*a*) pH 6.5 and (*b*) pH 4.6.

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution shell.

	Habu IX-bp at pH 6.5	Habu IX-bp at pH 4.6
Space group	$P2_1$	$C2$
Unit-cell parameters		
a (Å)	60.72	134.12
b (Å)	63.51	37.83
c (Å)	66.91	55.83
β (°)	117.01	110.46
Wavelength (Å)	1.54 (Cu $K\alpha$)	1.54 (Cu $K\alpha$)
Resolution (Å)	54.1–1.72 (1.78–1.72)	36.2–2.29 (2.37–2.29)
Unique reflections	48268	12064
Redundancy	4.0 (3.1)	3.8 (3.7)
Completeness (%)	99.0 (92.9)	95.7 (89.8)
$I/\sigma(I)$	7.8 (2.6)	7.3 (2.3)
R_{merge}† (%)	7.1 (17.3)	8.6 (27.2)
V_M (Å^3 Da^{-1})	2.0	2.3
Solvent content (%)	37.9	46.1

† $R_{merge} = \sum_h \sum_i |I(h,i) - \langle I(h)\rangle| / \sum_h \sum_i I(h,i)$, where $I(h,i)$ is the intensity value of the ith measurement of h and $\langle I(h)\rangle$ is the corresponding mean value of $I(h)$ for all i measurements.

2.2. Measurement of fluorescence intensity

Ca^{2+}-induced changes in the intrinsic fluorescence of habu IX-bp were measured using a Hitachi Fluorescence Spectrophotometer F4500 (Hitachi, Japan) equipped with a thermostatted cell holder (298 K) as described previously (Sekiya *et al.*, 1995).

2.3. Crystallization

Crystallization experiments for habu IX-bp were conducted at pH 6.5 and 4.6 at 293 K. Initial screening was performed by the micro-batch method at pH 6.5 and by the sitting-drop method at pH 4.6 using only those reagents from Crystal Screen and Crystal Screen 2 (Hampton Research) that contain suitable buffers or organic solvents. Optimization of crystallization conditions was conducted by changing the parameters of the initial conditions.

2.4. Data collection

A crystal at pH 6.5 was mounted in a nylon loop (Hampton Research) and flash-frozen in an N_2 gas stream at 100 K after successive soaking for a few seconds in reservoir solutions additionally containing 10 and 20%(*w*/*v*) PEG 400. Diffraction data were collected at 100 K on a Rigaku R-AXIS IV^{++} imaging-plate detector mounted on a Cu $K\alpha$ rotating-anode generator (Ultrax-18, Rigaku) with Osmic Confocal mirrors operated at 50 kV and 100 mA. Data collection for a crystal at pH 4.6 was conducted using a Cu $K\alpha$ rotating-anode generator (MicroMax007, Rigaku) equipped with a Rigaku R-AXIS VII imaging-plate detector and Osmic Confocal mirrors operated at 40 kV and 20 mA. The data were processed using the program *CrystalClear* (Rigaku). Data-collection statistics are summarized in Table 1.

3. Results and discussion

Measurements of intrinsic fluorescence changes upon Ca^{2+} binding to habu IX-bp revealed the pH-dependence of the Ca^{2+}-binding characteristics as in the case of habu IX/X-bp (Sekiya *et al.*, 1995), where the apparent K_d values at pH 7.5, 6.5 and 4.6 were 5 and 250, 20 and 1700, and 50 and 3600 μ*M*, respectively. The details will be described elsewhere.

Initial screening of crystallization conditions for habu IX-bp at pH 6.5 resulted in a lead condition from solution No. 7 of Crystal Screen

2 [10%(*w*/*v*) PEG 1000 and 10%(*w*/*v*) PEG 8000]. Rod-shaped crystals at pH 6.5 were grown by refining the conditions and the best crystals were obtained by mixing 0.5 µl protein solution containing ∼6 mg ml^{-1} protein, 20 m*M* MES pH 6.5 and 1 m*M* $CaCl_2$ and 1.5 µl precipitant solution containing 10%(*w*/*v*) PEG 1000, 10%(*w*/*v*) PEG 8000 and 20 m*M* MES pH 6.5 under a thin layer of paraffin oil (Hampton Research). Crystals grew to maximum size with approximate dimensions of 0.5 × 0.1 × 0.1 mm within a week (Fig. 1*a*). These crystals diffracted to beyond 1.7 Å and data to 1.72 Å were processed. The crystals belong to the monoclinic space group $P2_1$ and the $R_{\rm merge}$ of the data is 7.1%. The Matthews coefficient $V_{\rm M}$ is calculated to be 2.0 Å^3 Da^{-1} assuming that two molecules are located in the asymmetric unit and this corresponds to a solvent content of 37.9% (Matthews, 1968). On the other hand, crystals of IX-bp at pH 4.6 appeared as clusters of small plates. By refining the initial condition [No. 13 of Crystal Screen 2; 0.2 *M* ammonium sulfate, 0.1 *M* sodium acetate and 30%(*w*/*v*) PEG MME 2000] suitable crystals were obtained within two weeks. The precipitant solution of the optimum crystallization condition consisted of 0.2 *M* ammonium sulfate, 0.1 *M* rubidium acetate and 30%(*w*/*v*) PEG MME 2000 and crystals suitable for X-ray diffraction studies were obtained using the sitting-drop vapour-diffusion method by mixing equal volumes of protein solution (∼10 mg ml^{-1}) and precipitant solution and equilibrating against 50 µl reservoir (precipitant) solution at 293 K. The thin plate-like crystals (0.2 × 0.2 × 0.05 mm; Fig. 1*b*) belonged to the monoclinic space group *C*2 and diffracted to 2.29 Å. The number of molecules in the asymmetric unit is assumed to be one based on the Matthews coefficient calculation ($V_{\rm M}$ = 2.3 Å^3 Da^{-1}), with a solvent content of 46.1%. The data-collection statistics are summarized in Table 1. Structural analyses of both crystals are under way using the molecular-replacement method.

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