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Purification, crystallization and preliminary crystallographic analysis of AHP IX-bp, a zinc ion and pH-dependent coagulation factor IX binding protein from *Agkistrodon halys* Pallas venom

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A new coagulation factor IX binding protein, AHP IX-bp, has been purified from *Agkistrodon halys* Pallas venom and estimated to be an *AB* heterodimer of about 25 kDa consisting of two chains (an *A* chain of 15.5 kDa and a *B* chain of 15 kDa) linked by one or more disulfide bonds. The N-terminal sequence of AHP IX-bp has been determined and aligned with C-type lectin-like proteins. The protein has a high sequence similarity to some snake-venom C-type lectin-like proteins. AHP IX-bp binds to coagulation factor IX but not to coagulation factor X. Moreover, AHP IX-bp shows binding to coagulation factor IX in both zinc ion-dependent and pH-dependent manners. The affinity between AHP IX-bp and coagulation factor IX is higher under neutral or weakly alkaline conditions than under weakly acidic conditions. Single crystals of AHP IX-bp grown at pH 6.5 and 7.5 diffract X-rays to 2.0 and 1.8 Å resolution, respectively. Both crystals are isomorphous and belong to the space group *P*1; only one *AB* heterodimer is present in the unit cell.

1. Introduction

Coagulation factors IX and X play essential roles in the cascade reactions of blood coagulation, in which the two factors are sequentially activated, leading to the formation of insoluble fibrin clots from the soluble fibrinogen (Jackson & Nemerson, 1980). Their structures are homologous to each other: a Gla domain is located at the N-terminus followed by two growth-factor domains, one activation peptide or connecting domain and one catalytic domain (Yoshitake *et al.*, 1985; Leytus *et al.*, 1986). A family of coagulation factor IX/X binding proteins (abbreviated IX/X-BPs) has been identified in snake venoms and contains many peptides and proteins that affect haemostasis and thrombosis (Markland, 1998; Matsui *et al.*, 2000; Andrews & Berndt, 2000; Atoda & Morita, 1989; Atoda *et al.*, 1995, 1998; Sekiya *et al.*, 1993; Chen & Tsai, 1996; Xu *et al.*, 2000). Furthermore, this family belongs to a superfamily termed the snake-venom C-type lectin-like proteins. In addition to IX/X-BPs, α-thrombin-binding proteins (Zingali *et al.*, 1993), vWF-binding proteins (Andrews *et al.*, 1989; Hamako *et al.*, 1996) and platelet membrane glycoprotein-binding proteins (Peng *et al.*, 1991; Taniuchi *et al.*, 1995; Andrews *et al.*, 1996) have also been found to belong to the superfamily. IX/X-BPs of snake venoms can be further grouped into three classes according to their abilities to bind to coagulation factors IX or X or both (named IX-bp, X-bp and IX/X-bp, respectively) .

Interactions between the coagulation factors (IX and X) and a series of snake-venom IX/X-BPs, including habu IX-bp, habu IX/X-bp, *Deinagkistrodon acutus* X-bp, ACF II and ECLV IX/X-bp, are often Ca^{2+}-dependent (Atoda *et al.*, 1995, 1998; Sekiya *et al.*, 1995; Xu & Liu, 2001; Chen & Tsai, 1996). Three IX/X-BP structures have been solved; in each binding to the coagulation factors was shown to be Ca^{2+}-dependent (Mizuno *et al.*, 1997, 1999, 2001). Further research needs to be carried out in order to delineate the role of ions in the formation of complexes between IX/X-BPs and coagulation factors. Moreover, formation of the complex of habu IX/X-bp with coagulation factor IX or X takes place in a pH-dependent manner. The binding affinity is higher under neutral or weakly basic conditions than under weakly acidic conditions (Sekiya *et al.*, 1995). The structural basis of such pH-dependent interactions is still unknown and remains to be clearly elucidated.

The purpose of this paper is to report the purification and partial characterization of AHP IX-bp, a new coagulation factor IX binding protein from *A. halys* Pallas venom, for which the binding affinity to the target is both pH-dependent and Zn^{2+}-dependent. Single crystals of AHP IX-bp suitable for X-ray diffraction have been obtained under different pH conditions, leading to a good start

towards understanding the pH-dependent binding characteristics.

2. Materials and methods

2.1. Materials

Lyophilized venom of *A. halys* Pallas was obtained from the southern mountain region (Anhui, China). DEAE-Sepharose, CM-Sepharose, Sephacryl S-100 and streptavidin-biotinylated horseradish peroxidase complex (SBHP) were purchased from Pharmacia (Sweden). The Crystal Screen kit was obtained from Hampton Research (USA). Bovine coagulation factor X, trifluoroacetic acid (TFA), 4-vinylpyridine, *o*-phenylenediamine dihydrochloride (OPD), bovine serum albumin (BSA), Tween 20 and the immunoprobe biotinylation kit were obtained from Sigma (USA). Human coagulation factor IX was purchased from Merck (Germany). The Delta-Pak C4 column was obtained from Waters (USA). Standard proteins for molecular-weight estimation were produced by Shanghai Biochemical Technology Co. (China). Other reagents and chemicals were of analytical grade from commercial sources.

2.2. Preparation of native AHP IX-bp and its reduced *A* chain and *B* chain

Native AHP IX-bp was purified from the lyophilized crude venom of *A. halys* Pallas by three liquid chromatography steps (see Figs. 1*a*, 1*b* and 1*c*). AHP IX-bp was reduced and *S*-pyridylethylated according to the method of Atoda *et al.* (1991) with slight modification (see Fig. 1*d*).

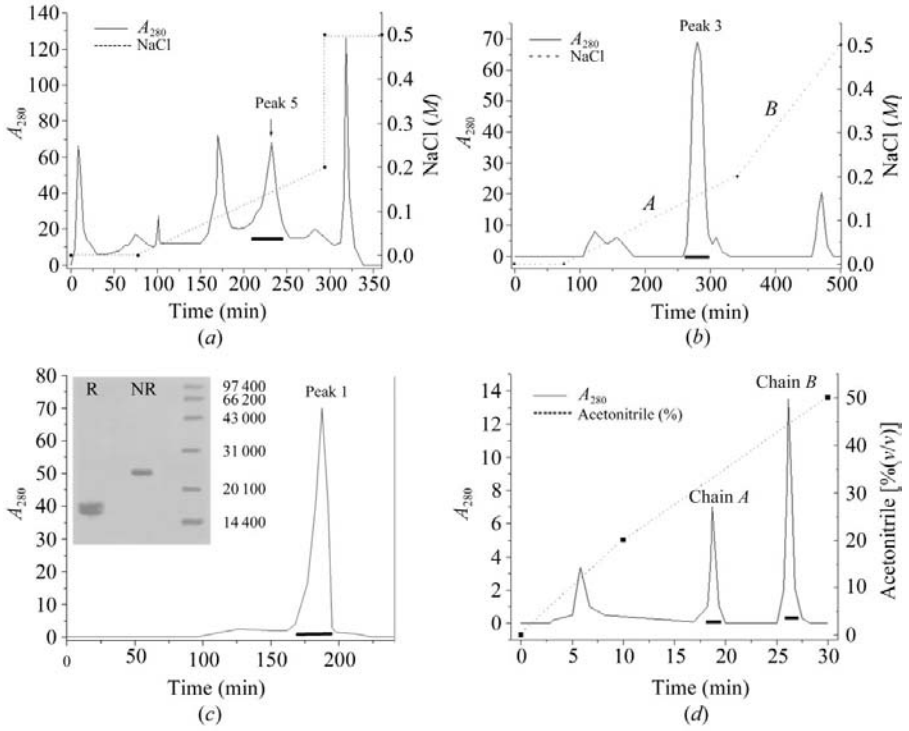

Figure 1
Preparation of native AHP IX-bp and its reduced *A* and *B* chains. (*a*) 1.5 g of crude venom from *A. halys* Pallas was dissolved in 30 ml of loading buffer *A* (0.02 *M* Tris–HCl pH 8.0) and centrifuged at 12 000 rev min^{-1} for 20 min. The supernatant was applied to a DEAE-Sepharose Fast Flow column (1.6 × 40 cm) pre-equilibrated with loading buffer *A*. The column was washed with the loading buffer *A* for 80 min at a flow rate of 180 ml h^{-1} and then eluted with 800 ml of a linear NaCl gradient from 0.0 to 0.2 *M* (in loading buffer *A*). Finally, the column was eluted with 100 ml of 0.02 *M* Tris–HCl buffer pH 8.0 containing 0.5 *M* NaCl. Peak 5, which contained AHP IX-bp, was pooled. (*b*) The fraction pooled from the previous column was dialyzed against loading buffer *B* (0.02 *M* NaOAc/HOAc pH 5.0) overnight at 277 K and then applied to a CM-Sepharose Fast Flow column (1.6 × 40 cm) pre-equilibrated with loading buffer *B*. The column was eluted with 800 ml of a linear NaCl gradient *A* from 0.0 to 0.2 *M* (in loading buffer *B*) and then with 400 ml of another linear NaCl gradient *B* from 0.2 to 0.5 *M* (in loading buffer *B*) at a flow rate of 160 ml h^{-1}. Peak 3 was pooled. (*c*) The pooled sample was concentrated and applied to a Sephacryl S-100 column (1.6 × 100 cm). The column was eluted with 0.15 *M* NaCl solution at a flow rate of 40 ml h^{-1}. Peak 1 was collected. The inset shows 15% SDS–PAGE analysis of purified AHP IX-bp carried out under non-reducing (NR) or reducing (R) conditions with 0.1 *M* β-mercaptoethanol. The right lane contains standard protein molecular-weight markers. (*d*) 3.2 mg AHP IX-bp was dissolved in 0.5 ml of 0.5 *M* Tris–HCl buffer pH 8.0 containing 6 *M* guanidine hydrochloride and 2 m*M* EDTA. 7 mg of dithiothreitol was added to the protein solution and the mixture was incubated for 2.5 h at 323 K. After addition of 4-vinylpyridine to a 3:1 molar ratio of 4-vinylpyridine:dithiothreitol, the mixture was allowed to stand for a further 2.5 h at 323 K and then dialyzed against distilled water and 0.1% TFA solution. Finally, the mixture was applied to a Delta-Pak C_4 column (3.9 × 150 mm) and sequentially eluted at room temperature with two linear gradients of acetonitrile [the acetonitrile concentration ranges were 0–20 and 20–50%(*v*/*v*), respectively, containing 0.1% TFA].

2.3. Assays for the binding of AHP IX-bp to the immobilized coagulation factor IX

AHP IX-bp was first biotinylated using an immunoprobe biotinylation kit; binding assays of AHP IX-bp to the immobilized coagulation factor IX were then carried out according to the method of Chen & Tsai (1996) with slight modification (see Fig. 2).

2.4. Crystallization

The protein pooled from liquid chromatography was desalted and concentrated to about 30 mg ml^{-1}. The absorbance at 280 nm was utilized to estimate the protein concentration, using an extinction coefficient ($A_{280}^{1\%}$) of 26.6. Using the typical hanging-drop vapour-diffusion method at room temperature (about 293–298 K), crystallization conditions were initially screened using the sparse-matrix sampling method (Jancarik & Kim, 1991). 2 µl of protein solution without any additives was mixed with 2 µl of reservoir solution (Crystal Screen and Crystal Screen II) and then equilibrated against 300 µl of reservoir solution. Microcrystals appeared one week later from condition No. 38 of the Crystal Screen kit, containing 1.4 *M* sodium citrate pH 7.5. Further improvement of crystallization conditions was performed to obtain better single crystals.

By mixing 2 µl of protein solution (20 mg ml^{-1} in double-distilled water) with 2 µl of reservoir solution (1.4 *M* sodium citrate pH 7.5) and then equilibrating against the reservoir solution for three weeks, crystals could be obtained at weakly alkaline pH values (Fig. 3*a*). However, growing crystals at weakly acidic pH values was more difficult. 2 µl of protein solution (20 mg ml^{-1} in double-distilled water) was mixed with 2 µl of reservoir solution (1.4 *M* sodium citrate pH 6.5) and equilibrated against 300 µl of reservoir solution. After the microcrystals appeared one week later, they were transferred to drops made up of a mixture of 2 µl of protein solution (20 mg ml^{-1} in double-distilled water) and 2 µl of reservoir solution (1.2 *M* sodium citrate pH 6.5) and equilibrated for one week. Single crystals suitable for X-ray diffraction (Fig. 3*b*) appeared in two weeks.

Table 1
Statistics of diffraction data collection and reduction.

Values in parentheses are for the highest resolution shell.

Crystallization pH value	7.5	6.5
Space group	*P*1	*P*1
Unit-cell parameters		
a (Å)	31.58	31.61
b (Å)	46.54	46.57
c (Å)	52.20	52.44
α (°)	94.52	94.16
β (°)	101.61	101.44
γ (°)	100.61	100.47
No. of observations	24472	17989
No. of independent reflections	12524	9084
Resolution limits (Å)	20–1.8 (1.84–1.8)	20–2.0 (2.05–2.0)
$R_{\rm merge}$† (%)	5.7 (27.2)	7.5 (14.3)
Completeness‡ (%)	92.9 (87.6)	93.0 (79.5)

† $R_{\rm merge} = \sum_h \sum_j |\langle I(h)\rangle - I(h)_j| / \sum_h \sum_j I(h)_j$, where $I(h)_j$ is the *j*th observed reflection intensity and $\langle I(h)\rangle$ is the mean intensity of reflection *h*. ‡ The completeness is the ratio of number of observed reflections to the number of possible reflections.

2.5. Collection and reduction of X-ray diffraction data

Using similar conditions, X-ray diffraction data from crystals grown under both weakly acidic and alkaline conditions were collected at room temperature. For each type of single crystal, a total of 180 imaging frames was recorded at a crystal-to-detector distance of 120 mm using a MAR Research imaging plate (diameter 300 mm) mounted on an X-ray generator with a graphite monochromator and a sealed copper-target tube. A 1° oscillation angle, 900 s exposure time per imaging frame and a tube voltage/current of 40 kV/50 mA were utilized. The diffraction data were processed using *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997). The statistics of data collection and reduction are listed in Table 1.

2.6. Miscellaneous

Native PAGE was utilized to examine the formation of complexes of AHP IX-bp and coagulation factors. Sequencing of the N-terminal amino-acid residues was performed using the Edman degradation method at The Normal University of Hunan, China. The metal-ion content in the protein samples was analyzed using an atomic absorption spectrophotometer (AA-3510; The 4th Analytic Instrument Factory, Shanghai, China).

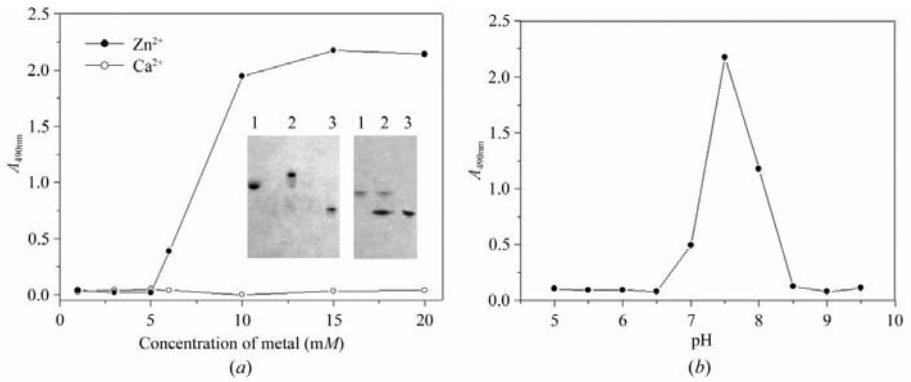

Figure 2
Binding of AHP IX-bp to coagulation factors. 2 mg lyophilized AHP IX-bp was dissolved in 1 ml of 0.1 *M* phosphate buffer pH 7.2 and mixed with 19.5 µl BAC-SulfoNHS (biotinamidocaproate *N*-hydroxysulfosuccinimide) 10 mg ml^{-1} in 0.1 *M* phosphate buffer pH 7.2) with gentle stirring. The mixture was then incubated with gentle stirring for 30 min at room temperature. The biotinylated AHP IX-bp was purified by chromatography on a Sephadex G-25M column (1.5 × 5 cm) pre-equilibrated with PBS buffer (0.1 *M*, pH 7.2) (data not shown). Each well of the 96-well plate was coated with 50 µl of coagulation factor IX (dissolved in 50 n*M* $NaHCO_3$ buffer pH 9.4) at 277 K overnight. The plate was blocked with 350 µl of 1% BSA in TBST (20 m*M* Tris–HCl pH 7.5 containing 140 m*M* NaCl and 0.05% Tween 20) for 1 h at room temperature. After washing the plate three times with 350 µl TBST, 50 µl of 100 n*M* biotinylated AHP IX-bp (in different buffers according to the goals, see detailed descriptions below) was added and incubated for 1 h at room temperature. The plate was washed again three times with 350 µl TBST. 1000-fold diluted SBHP was then added and incubated at 310 K for 1 h. The plate was again washed three times with TBST; 0.4 mg ml^{-1} OPD pH 5.0 and 0.012% H_2O_2 pH 5.0 were then added. The reaction was stopped after 30 min by addition of 3 *M* H_2SO_4. The absorbance at 490 nm was measured using a microplate reader (ELx 800, Bio-Tek). (*a*) The biotinylated AHP IX-bp was in TBS buffer (20 m*M* Tris–HCl pH 7.5 containing 140 m*M* NaCl and different concentrations of $ZnSO_4$ or $CaCl_2$) The inset shows native PAGE assays examining the formation of complexes of AHP IX-bp with the coagulation factors. In the presence of 15 m*M* $ZnSO_4$, AHP IX-bp and coagulation factors (IX or X) were incubated at 310 K for 5 min in 50 m*M* Tris–HCl buffer pH 7.5. The mixture was then subjected to a 8% native PAGE gel to examine complex formation. Left-hand gel: lane 1, 100 n*M* factor IX; lane 2, 100 n*M* factor IX pre-incubated with 100 n*M* AHP IX-bp in the presence of 15 m*M* $ZnSO_4$; lane 3, 100 n*M* AHP IX-bp. Right-hand gel: lane 1, 50 n*M* factor X; lane 2, 50 n*M* factor X pre-incubated with 200 *M* AHP IX-bp in the presence of 15 m*M* $ZnSO_4$; lane 3, 200 n*M* AHP IX-bp. (*b*) The biotinylated AHP IX-bp was in solutions containing 15 m*M* $ZnSO_4$ and buffered at different pH values. The pH 5–5.5, 6.5, 7.0, 7.5–8.5 and 9.0–9.5 solutions were buffered using sodium acetate, MES, HEPES, Tris–HCl and glycine–NaOH, respectively.

3. Results and discussion

A new protein purified from *A. halys* Pallas venom has been estimated to be a heterodimer of about 25 kDa consisting of two chains (an *A* chain of 15.5 kDa and a *B* chain of 15 kDa) linked by disulfide bond(s) (see inset in Fig. 1*c*). When aligned using the software package *ClustalW* (Thompson *et al.*, 1994), the sequence of the N-terminal amino-acid residues of the reduced *A* chain and *B* chain shows a high homology to some snake-venom C-type lectin-like proteins (see Fig. 4), indicating that the purified protein is a new member of this superfamily. The complex of the protein and coagulation factor IX could be detected in the presence of Zn^{2+} ion (see inset in Fig. 2*a*); however, a complex of the protein and coagulation factor X did not form under similar conditions. Moreover, no effect of the protein on the clotting time of fibrinogen could be detected (data not shown), *i.e.* it may lack the ability to bind to α-thrombin. Clearly, it is still necessary to check for binding to vWF and platelet membrane glycoproteins. To our knowledge, none of the members of the family of snake-venom IX/X-BPs possess two different kinds of binding targets. Therefore, the protein reported here is designated AHP IX-bp.

Zinc and calcium ions have been found in AHP IX-bp samples. The molar ratio of protein molecule, calcium ion and zinc ion is about 1:1:1 (data not shown). It is reasonable to presume that two ionic binding sites might exist in AHP IX-bp molecules: one site for a zinc ion and another for a calcium ion. Similar situations appear in other known snake-venom IX/X-BPs, including habu IX/X-bp, habu IX-bp, ECLV IX/X-bp, *D. acutus* X-bp and ACF II, in which two calcium ion binding sites with different affinities were located (Sekiya *et al.*, 1995; Atoda *et al.*, 1995, 1998; Chen & Tsai, 1996; Xu & Liu, 2001). However, none of them has been reported to be capable of binding a zinc ion.

A series of divalent metal ions have been screened quantitatively to investigate their effects on the bindings of AHP IX-bp to coagulation factor IX. AHP IX-bp binds to the coagulation factor in a zinc ion dependent manner (see Fig. 2*a*). In the absence of Zn^{2+} ion, AHP IX-bp no longer binds to factor IX. The Ca^{2+} ion (also see Fig. 2*a*) as well as other metal ions (such as Mg^{2+}, Ba^{2+}, Sr^{2+} and Cu^{2+} ions, data not shown) has no effect on this type of binding. These results are different from those observed for the homologous proteins mentioned above. Habu IX-bp, habu IX/X-bp, *D. acutus* X-bp,

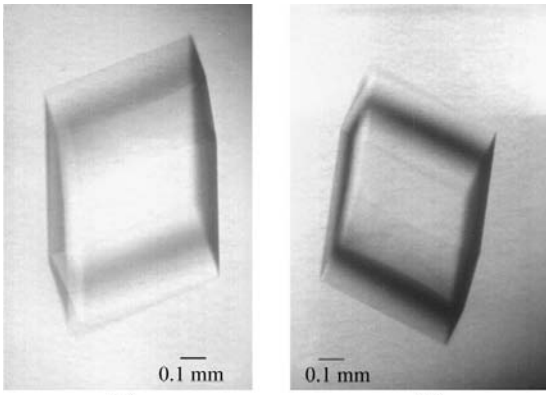

Figure 3
Crystals of AHP IX-bp grown at (*a*) pH 7.5 and (*b*) pH 6.5.

A chain	1 5 10 15
AHP IX-bp	--DCPSGWSSYEGHCYQTF---
Habu IX-bp	--DCPSGWSSYEGHCYKPFKLYKTWDDAERFC---
Habu IX/X-bp	--DCLSGWSSYEGHCYKAFEKYKTWEDAERVC---
D. acutus X-bp	--DCSSGWSSYEGHCYKVFKQSKTWADAESFC---
Botrocetin	--DCPSGWSSYEGNCYKFFQQKMNWADAERFC---
Flavocetin-A	DFDCIPGWSAYDRYCYQAFSKPKNWEDAESFC---
B chain	1 5 10 15
AHP IX-bp	--DCPSGWSSYEGHCYKPF---
Habu IX-bp	--DCPSDWSSYEGHCYKPFSEPKNWA---
Habu IX/X-bp	--DCPSDWSSYEGHCYKPFSEPKNWA---
D. acutus X-bp	--DCPSDWSSYEGHCYKPFNEPKNWA---
Botrocetin	--DCPPDWSSYEGHCYRFFKEWMHWD---
Flavocetin-A	GFCCPLGWSSYDEHCYQVFQQKMNWE---

Figure 4
N-terminal sequence alignment of AHP IX-bp and some C-type lectin-like proteins from snake venoms. Habu IX-bp and habu IX/X-bp are coagulation factor IX binding protein and factor IX/X binding protein from *Trimeresurus flavoviridis* venom, respectively (Atoda *et al.*, 1995; Matsuzaki *et al.*, 1996). *D. acutus* X-bp is a coagulation factor X-binding protein from *D. acutus* venom (Atoda *et al.*, 1998). Botrocetin is a vWF-binding protein from *Bothrops jararaca* venom (Usami *et al.*, 1993). Flavocetin-A is a platelet GPIb-binding protein from *T. flavoviridis* venom (Shin *et al.*, 2000). The sequence of AHP IX-bp was determined by the Edman degradation method.

ACF II and ECLV IX/X-bp all display Ca^{2+}-dependent ligand binding; ECLV IX/X-bp still binds to coagulation factor IX in the presence of Sr^{2+} (Chen & Tsai, 1996).

AHP IX-bp also binds to coagulation factor IX in a pH-dependent manner (see Fig. 2*b*). The optimum binding affinity appears to be around pH 7.5. A decrease in pH value from 7.5 to 6.5 causes a dramatic loss of its binding activity. Similarly, habu IX/X-bp has also been found to bind coagulation factors in a pH-dependent manner (Sekiya *et al.*, 1995), with optimum pH values for binding to coagulation factor IX and factor X of pH 8.0–8.5 and pH 7.5, respectively. It loses this binding ability below pH 6.0.

In order to elucidate the structural basis of pH-dependent binding characteristics, the X-ray diffraction data of AHP IX-bp crystals grown at pH 6.5 and 7.5 have been collected and reduced. The crystals are isomorphous and belong to space group $P1$. The V_M value (Matthews, 1968) is about 2.9 $Å^3$ Da^{-1}, corresponding to the presence of only one heterodimer in the unit cell. The crystal structures of three known IX/X-BPs (habu IX/X-bp, habu IX-bp and *D. acutus* X-bp) belong to space groups $P2_12_12$, $P2_1$ and $P4_12_12$, respectively (Mizuno *et al.*, 1997, 1999, 2001). Therefore, further structural studies on AHP IX-bp may reveal a new type of packing mode in the crystals. The sequencing of the complete amino-acid residue sequence and the the solution of the pH-dependent crystal structures of AHP IX-bp are being carried out in our laboratory.

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