Calcium-binding analysis and molecular modeling reveal echis coagulation factor IX/factor X-binding protein has the Ca-binding properties and Ca ion-independent folding of other C-type lectin-like proteins

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Received 1 August 2002; revised 24 September 2002; accepted 25 September 2002

First published online 10 October 2002

Edited by Robert B. Russell

Abstract **Many biologically active heterodimeric proteins of snake venom consist of two C-type lectin-like subunits. One of these proteins, habu IX/X-bp, is a Gla domain-binding protein whose subunits both bind to a Ca^{2+} ion, with a total of two Ca^{2+}-binding sites. The molecular modeling and Ca^{2+}-binding analysis of echis IX/X-bp revealed that it lacks one of two Ca^{2+}-binding sites, though the folding of this subunit is conserved. It is concluded that heterodimeric C-type lectin-like proteins function independent of Ca^{2+} and have essentially a similar folding to habu IX/X-bp.**

Key words: Anticoagulant protein; Snake venom; Three-dimensional model; Ca^{2+}-binding; *Echis carinatus leucogaster*; C-type lectin

1. Introduction

The anticoagulant proteins habu IX-bp (factor IX-binding protein from *Trimeresurus flavoviridis* venom) and habu IX/X-bp (factor IX/factor X-binding protein from *T. flavoviridis* venom) and acutus X-bp (factor X-binding protein from *Deinagkistrodon acutus* venom) are disulfide-linked heterodimers of C-type lectin-like subunits [1–3]. These proteins bind to γ-carboxyglutamic acid (Gla)-containing domains of coagulation factors IX and X in the presence of calcium ions [2–4]. The crystal structures of habu IX/X-bp, habu IX-bp, and acutus X-bp revealed that each subunit of these proteins has a Ca^{2+}-binding site different from that of C-type lectins such as mannose-binding protein and the central loop of each subunit is swapped to each other for dimerization [5–7]. The crystal structures of habu IX/X-bp, habu IX-bp, and acutus X-bp [5–7] also revealed that these proteins have Ca^{2+}-binding sites made up by Ser41, Glu43, Glu47, and Glu128 in subunit A (Fig. 1), and by Ser41, Gln43, Glu47, and Glu120 in subunit B (Fig. 2). These eight amino acid residues forming the Ca^{2+}-binding sites of these three C-type lectin-like proteins were totally conserved.

Many venom proteins consist of one or more heterodimers of C-type lectin-like subunits. These proteins include platelet glycoprotein Ib-binding aggregation inducer, alboaggregin B [8] and platelet aggregation inhibitors such as flavocetin A, echicetin, and agkicetin [9–11]. Echicetin complexed with IgM κ induces platelet agglutination and activation [12]. This protein family also contains other platelet aggregation inducers such as botrocetin [13], bitiscetin [14], rhodocytin/aggretin [15,16] and convulxin [17], and the thrombin inhibitor, bothrojaracin [18]. Their biological activity is reported to be Ca^{2+}-independent in contrast with anticoagulant proteins.

Chen and Tsai isolated ECLV IX/X-bp (echis IX/X-bp), factor IX/factor X-binding protein from the venom of *Echis carinatus leucogaster* and sequenced its entire length [19]. The apparent dissociation constants for the binding of echis IX/X-bp to factor IX and factor X were 6.6 and 125 nM, respectively, in the presence of Ca^{2+} ion [19]. In subunit A of echis IX/X-bp, four corresponding amino acid residues of habu IX/X-bp subunit A, Ser41, Glu43, Glu47, and Glu128, forming the Ca^{2+}-binding site, were conserved (Fig. 1). Subunit B of echis IX/X-bp has, however, Arg43 and Lys121 instead of Gln43 and Glu120 of habu IX/X-bp subunit B, suggesting that subunit B of echis IX/X-bp has no Ca^{2+}-binding site (Fig. 2).

Here we investigated the Ca^{2+}-binding characteristics of echis IX/X-bp and established a molecular model of echis IX/X-bp using the crystal structure of habu IX/X-bp to know the structure and Ca^{2+}-binding relationship for IX/X-bp function using echis IX/X-bp as a model. We will discuss the difference in the calcium-binding properties of IX/X-bps and related proteins based on a comparison of the amino acid sequence and three-dimensional structure of IX/X-bps and other C-type lectin-like proteins. We will also suggest a way to estimate the Ca^{2+}-binding ability of these proteins.

2. Materials and methods

2.1. Isolation of echis IX/X-bp

Echis IX/X-bp was isolated by the method of Chen and Tsai [19]

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Abbreviations: echis IX/X-bp, factor IX/factor X-binding protein from *Echis carinatus leucogaster* venom; habu IX-bp, factor IX-binding protein from *Trimeresurus flavoviridis* venom; habu IX/X-bp, factor IX/factor X-binding protein from *Trimeresurus flavoviridis* venom; acutus X-bp, factor X-binding protein from *Deinagkistrodon acutus* venom; C-type lectin

0014-5793/02/$22.00
PII: S0014-5793(02)03507-X

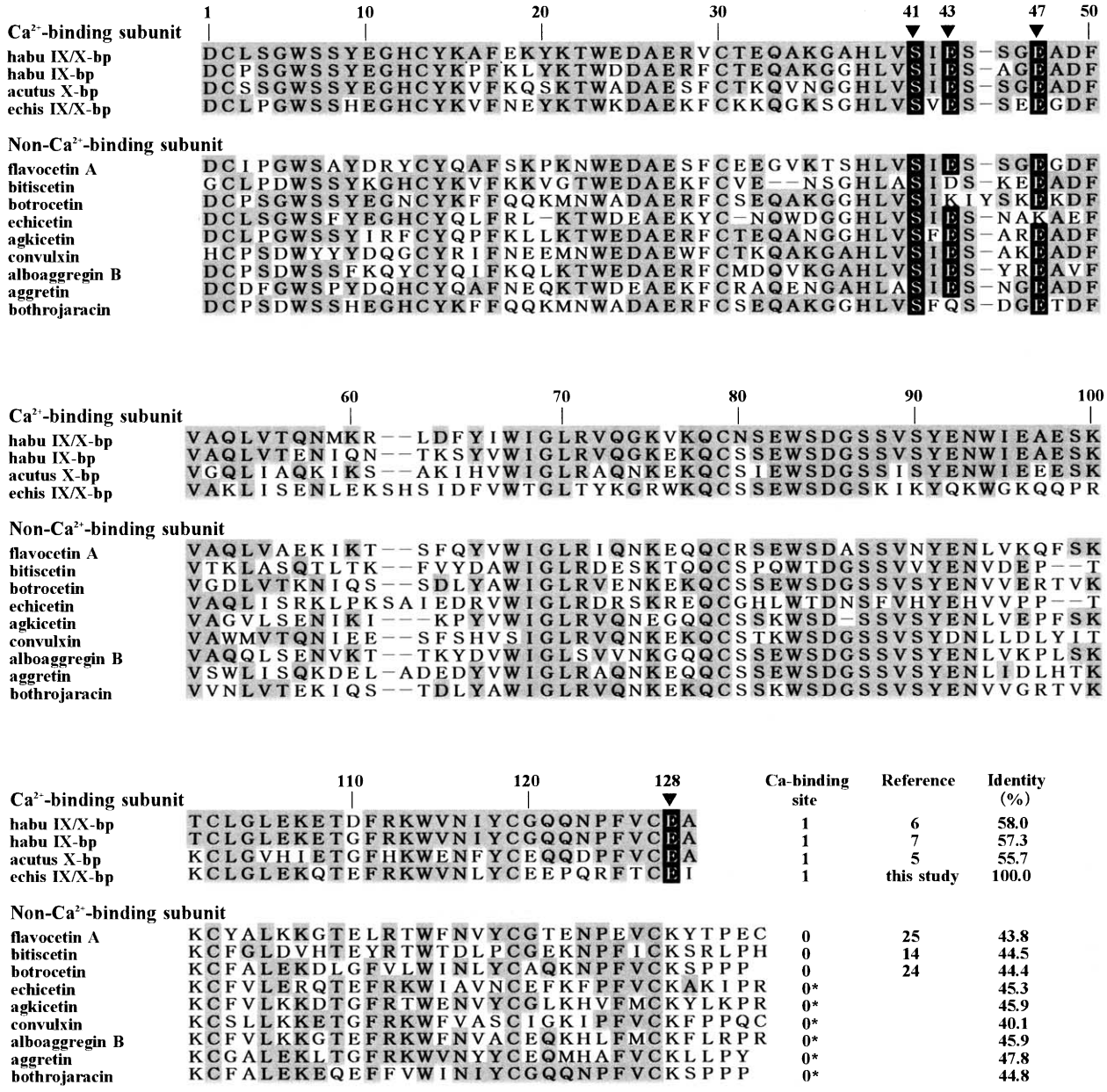

Fig. 1. Comparison between subunit A of IX/X-bps and structurally related proteins. Only residues that are conserved between habu IX/X-bp and any of the other proteins are shaded. Ca^{2+} ligands in habu IX/X-bp are marked with ▼ and reversed characters indicate amino acid residues at the corresponding Ca^{2+}-binding site with Ca^{2+}-binding ability. * indicates number of potential Ca^{2+}-binding sites. Identities between echis IX/X-bp A subunit and various C-type lectin-like subunits are indicated.

with some minor modification from lyophilized venom of *E. carinatus leucogaster*. Its cross-reactivity to anti-habu IX/X-bp antibodies and activity to bind coagulation factors were tested by enzyme-linked immunosorbent assay (ELISA) using polyclonal antibodies against habu IX/X-bp as described previously [4]. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) of purified echis IX/X-bp gave a protein band with a M_r of 25 000 Da under non-reducing conditions, and polypeptide chains of M_r 15 500 and 12 500 Da under reducing conditions. The amino-terminal amino acid sequences of each subunit of purified protein were determined after SDS–PAGE and blotting on polyvinylidene difluoride (PVDF) membrane by the method of Hirano [20] using a protein sequencer (model 473A; Applied Biosystems, Foster City, CA, USA). The sequences of subunits A and B were DXLPGWSSHEGHXYKVFNEYK and DXSSGWTAYGKHXYXVFDEP (X denotes an unidentified amino acid), which were identical to the sequences of Chen and Tsai [19].

2.2. Equilibrium dialysis

Equilibrium dialyses of echis IX/X-bp (40 μM) and habu IX-bp (30 μM) were performed in 50 mM Tris–HCl buffer, pH 8.0, containing 0.1 M NaCl, as described previously [3]. A 150-μl aliquot of a solution of $CaCl_2$ that contained $^{45}CaCl_2$ as a tracer (100 000 dpm/cell; Dupont, New England Nuclear) was dialyzed against 150 μl of a protein solution for 20 h with constant rotation.

2.3. Homology modeling of echis IX/X-bp

Homology modeling of echis IX/X-bp was carried out as follows: (1) Habu IX/X-bp (PDB ID: 1IXX) [6] was selected as the most suitable template for the modeling. (2) The sequences were aligned as shown in Figs. 1 and 2. (3) Loop searching and loop replacement were done when insertion/deletion sites were required. (4) Side chains were replaced. (5) Steric hindrances between side chains were removed. Here, 100 000 random conformational searches for each side chain were carried out by using random numbers which reproduce the

normal distribution of dihedral angles (χ) experimentally found in crystals [21]. However, based on the three-dimensional structural fitting between habu IX/X-bp, habu IX-bp (PDB ID: 1BJ3) and acutus X-bp (PDB ID: 1IOD), the side chains structurally well conserved were fixed at positions equivalent to the side chains of habu IX/X-bp. For the amino acid residues Arg43 and Lys121 in subunit B which are not conserved, a further 1 000 000 random conformational searches were carried out by rotating the bonds of side chain atoms of these two residues. (6) Water molecules which were observed in the crystal structure of habu IX-bp were included in the calculation to minimize energy, and overlapping water molecules were removed during the calculation. Terminal and charged residues were assumed to be in their normal states. Cff91 and a fast multipole method were used as a force-field and for the calculation of long-range electrostatic interaction, respectively. Conjugate gradient optimization was carried out with a termination threshold for the maximum component of the energy gradients of 0.1 kcal/mol Å.

A program package for protein engineering and drug design, BIOCES[E] (NEC Corp., Japan) [22], was used for procedures (1)–(5). Energy minimization and display of the molecule were carried out using Discover and Insight (Accelrys Inc.), respectively. All the calculations were performed on O2 (Silicon Graphics).

2.4. Quality of the model

The stereochemistry of the protein was checked as follows. All the bond lengths and bond angles were found to be standard. No abnormal van der Waals contacts were found between main chain atoms, between side chain atoms, or between main chain atoms and side chain atoms. The geometry of the main chain dihedral angles (ϕ,ψ) had desirable values in the Ramachandran plot.

3. Results and discussion

The binding of $^{45}Ca^{2+}$ to echis IX/X-bp and habu IX-bp was investigated by equilibrium dialysis. Habu IX-bp had two

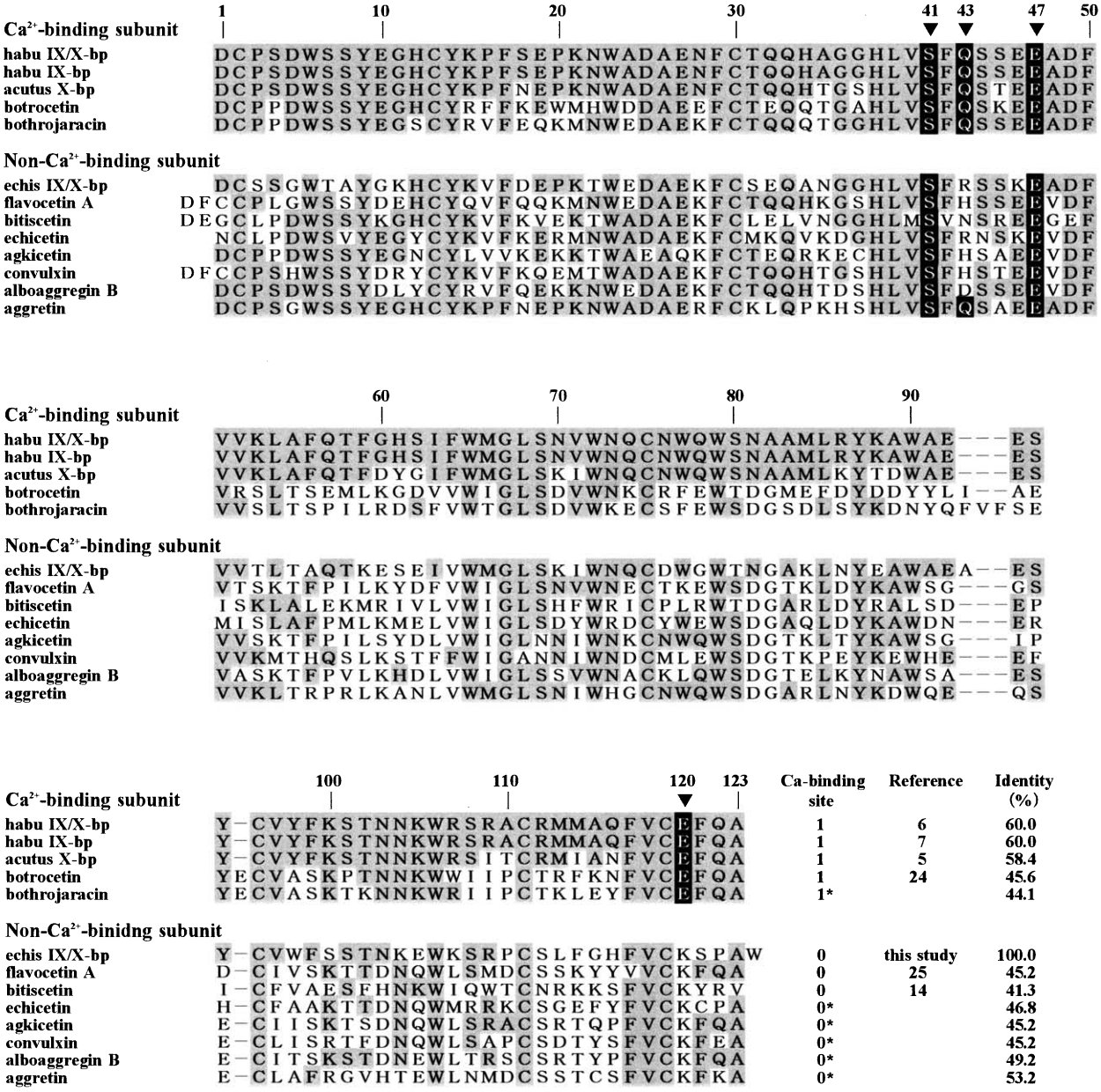

Fig. 2. Comparison between subunit B of IX/X-bps and structurally related proteins. For details, see legend to Fig. 1. In the case of botrocetin [24], Mg^{2+} ion was bound in subunit B, since crystallization was performed in the presence of 100 mM Mg^{2+}. Identities between echis IX/X-bp B subunit and various C-type lectin-like subunits are indicated.

independent Ca^{2+}-binding sites; a high affinity site with a K_d value of 16 ± 1 μM and a low affinity site with a K_d value of 109 ± 7 μM (Table 1). The present Ca^{2+}-binding properties of habu IX-bp were essentially consistent with the data for habu IX-bp, habu IX/X-bp, and acutus X-bp [2,3,23]. In contrast, echis IX/X-bp bound only one Ca^{2+} ion per molecule with a K_d value of 47 ± 4 μM (Table 1).

Fig. 3 shows the model of echis IX/X-bp prepared from the crystal structure of habu IX/X-bp [6]. Three-dimensional modeling revealed the overall structure of echis IX/X-bp to be similar to that of habu IX/X-bp. Root mean square deviations after superimposing Cα atoms except insertion regions are 0.3, 0.9 and 0.8 Å for habu IX/X-bp, habu IX-bp and acutus X-bp, respectively.

A notable feature is that echis IX/X-bp has a Ca^{2+}-binding site in subunit A, but not in subunit B. Gln43 and Glu120 in subunit B of habu IX/X-bp are replaced by positively charged Arg43 and Lys121 in echis IX/X-bp (Fig. 2). Although in overall structure, subunit B of echis IX/X-bp is essentially the same as that of habu IX/X-bp, it is most likely to have no ability to bind Ca^{2+} ion. Since echis IX/X-bp bound only one Ca^{2+} ion per molecule in this equilibrium dialysis experiment, a Ca^{2+} ion should bind to subunit A.

The crystal structures of other C-type lectin-like heterodimers have revealed Ca^{2+}-binding sites as follows: (1) botrocetin has a metal ion-binding site only in subunit B (Fig. 2), but in this case Mg^{2+} bound in place of Ca^{2+} [24]; (2) flavocetin A [25] and bitiscetin [14] have no Ca^{2+}-binding site. The upper panel of Fig. 4 shows the Ca^{2+}-binding site in subunit A of echis IX/X-bp superimposed on that of habu IX/X-bp for comparison. Four coordinating residues, Ser41, Glu43, Glu47, and Glu130 (Glu128 in habu IX/X-bp), are altogether conserved and these two structures are very similar. On the other hand, the subunit B of echis IX/X-bp has no complete coordinating residues, resulting in no Ca^{2+}-binding site (Fig. 4, middle panel). The NZ atom of Lys121 of subunit B is close to the position corresponding to the Ca^{2+} ion occupied in habu IX/X-bp, and contributes to the neutralization of negative charges and to the stabilization via hydrogen bonds of the OE atom of Glu47 and the OG atom of Ser41 (Fig. 4, middle panel). It is noteworthy that this structure is similar to that in the corresponding regions of flavocetin A subunit B [25], bitiscetin subunit B [14], and botrocetin subunit A [24] as shown in the lower panel of Fig. 4.

As shown in Fig. 1, the Ca^{2+}-binding subunit A of habu IX/X-bp, habu IX-bp, and acutus X-bp has Glu128, whereas the non-Ca^{2+}-binding subunit A of flavocetin A, bitiscetin, and botrocetin has a Lys residue in its place. Similarly, Glu120 of habu IX/X-bp subunit B is conserved in the

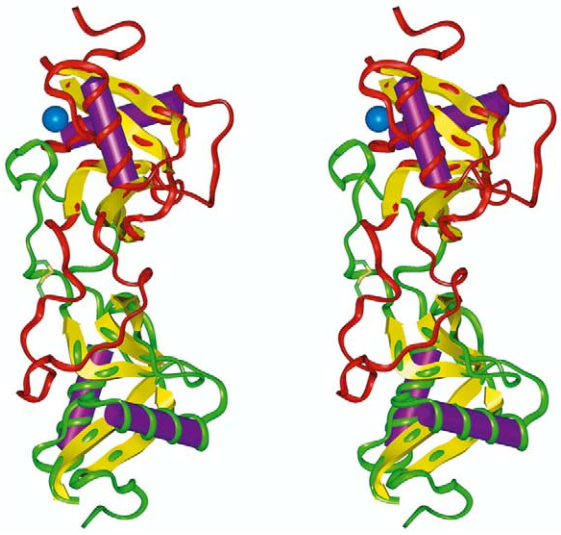

Fig. 3. Stereoview of the echis IX/X-bp model. Subunits A and B are shown in magenta and green, respectively, and the intersubunit disulfide bond is represented by a stick model. α-Helices and β-strands are shown as purple cylinders and yellow arrows. A blue sphere represents Ca^{2+} ion.

Ca^{2+}-binding subunit B of habu IX-bp, acutus X-bp, and botrocetin, but replaced with Lys in the non-Ca^{2+}-binding subunit B (Fig. 2). The folding of these subunits does not need Ca^{2+}-binding in the Ca^{2+}-binding area, because the Lys residue is inserted into the site corresponding to the position of the Ca^{2+} ion and forms the hydrogen bonds to stabilize IX/X-bp-like folding. The Lys residue in this position is thus structurally important and strictly conserved in non-Ca^{2+}-binding subunits. On the other hand, at position 43 (echis IX/X-bp numbering), a variety of residues are present in non-Ca^{2+}-binding subunits (Figs. 1 and 2). Gln43 in subunit B of habu IX/X-bp and the corresponding amino acid residues in other C-type lectin-like proteins are compared in Fig. 4 (lower panel). His45 in flavocetin A, and Asn45 in bitiscetin are flipped over and exposed to the solvent, while the side chain conformation of Arg43 in echis IX/X-bp takes a different orientation, forming a hydrogen bond between its NE atom and the OE atom of Glu47. A similar structure is observed in the corresponding region in subunit A of botrocetin, where the NZ atom of Lys43 forms a hydrogen bond with the OE atom of Glu48 [24] as shown in the lower panel of Fig. 4. Thus, the residue corresponding to Arg43 in subunit B of echis IX/X-bp takes a variety of conformations in non-Ca^{2+}-binding subunits, and does not contribute much to the stabilization of the structure. The crystal structure of bothro-

Table 1
Ca^{2+}-binding properties of echis IX/X-bp, habu IX-bp, habu IX/X-bp, and acutus X-bp

Protein	Binding sites	K_d values (mean ± S.E.M.) (μM)		
		High affinity	Low affinity	*n*
Echis IX/X-bp	1	47 ± 4	–	3
Habu IX-bp	2	16 ± 1	109 ± 7	3
Habu IX-bp[a]	2	14 ± 4	130 ± 100	7
Habu IX/X-bp[b]	2	25 ± 12	202 ± 110	5
Acutus X-bp[c]	2	16 ± 1	103 ± 10	6

[a]From [2].
[b]From [23].
[c]From [3].

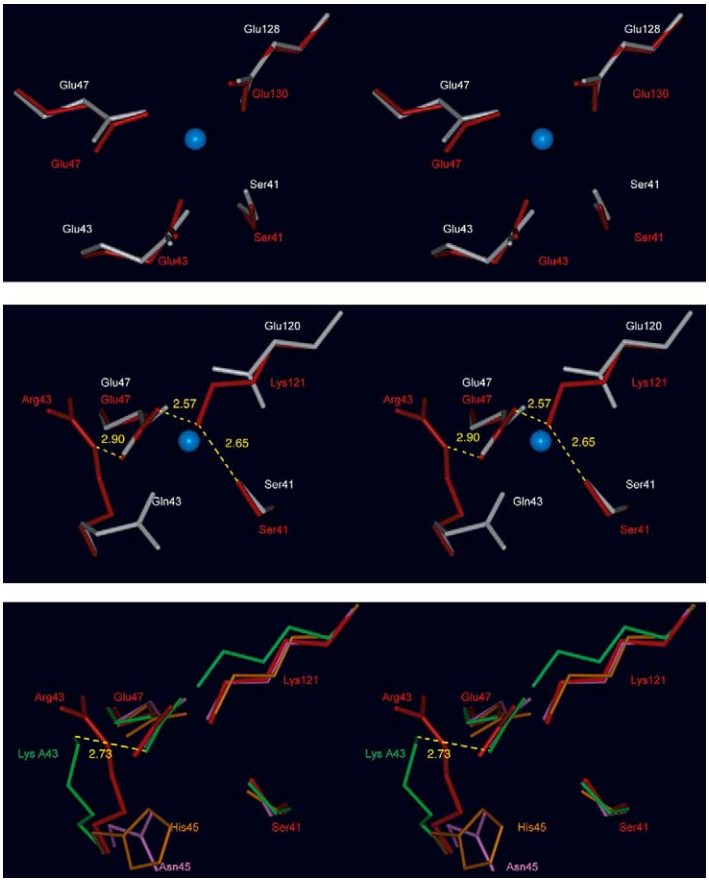

Fig. 4. Stereoviews of superpositions of Ca^{2+}-binding sites and equivalent regions. Upper panel: Stereoview of the superposition of the Ca^{2+}-binding site of habu IX/X-bp subunit A (white) and the equivalent region of echis IX/X-bp subunit B (red). A blue sphere represents Ca^{2+} ion in habu and echis IX/X-bps. Middle panel: Stereoview of the superposition of the Ca^{2+}-binding site of habu IX/X-bp subunit B (white) and the equivalent region of echis IX/X-bp subunit B (red). A blue sphere represents Ca^{2+} ion in habu IX/X-bp. Lower panel: The same view as in the middle panel, but the equivalent regions of structurally known proteins where Ca^{2+} ions are not bound are superimposed on echis IX/X-bp. These proteins are echis IX/X-bp subunit B (red), flavocetin A subunit B (brown), bitiscetin subunit B (purple), and botrocetin subunit A (green).

jaracin is not known, but it is suggested that subunit A lacks Ca^{2+} ion-binding site whereas subunit B binds Ca^{2+} ion based on the sequence comparison (Figs. 1 and 2).

In summary, echis IX/X-bp had only one Ca^{2+}-binding site in subunit A. A three-dimensional molecular model shows that subunit A binds a Ca^{2+} ion and subunit B has no Ca^{2+}-binding site. The molecular model of echis IX/X-bp subunit B is quite similar to the structures of flavocetin A and bitiscetin determined by X-ray crystallography. The present study indicates that, even in IX/X-bp-like proteins containing the subunit lacking any Ca^{2+} ligands, the main chain fold is maintained as the case of the IX/X-bp-like anticoagulant proteins.

Acknowledgements: This work was supported in part by a grant-in-aid for Scientific Research from the Ministry of Education, Science and Culture of Japan (T.M.), by a grant from the Welfide Medicinal Research Foundation, and by a Special Grant from Meiji Pharmaceutical University (H.A.), as well as by Special Coordination Funds for Promoting Science and Technology (H.M.).

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