A novel dimer of a C-type lectin-like heterodimer from the venom of *Calloselasma rhodostoma* (Malayan pit viper)

Runhua Wang[a], Chunguang Kong[a], Prasanna Kolatkar[b,c], Maxey C.M. Chung[a,d,*]

[a] *Department of Biochemistry, National University of Singapore, Singapore, Singapore*
[b] *Institute of Molecular and Cell Biology, National University of Singapore, Singapore, Singapore*
[c] *Bioinformatics Centre, National University of Singapore, Singapore, Singapore*
[d] *Bioprocessing Technology Centre, National University of Singapore, Singapore, Singapore*

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Abstract **We have isolated a potent platelet aggregation inducer from the crude venom of *Calloselasma rhodostoma* (Malayan pit viper), termed rhodoaggretin, with a novel oligomeric structure consisting of a dimer of C-type lectin-like heterodimers. On the basis of its native molecular mass of 66 kDa, and a M_r of 30 kDa for its disulfide-linked αβ-heterodimer, we propose that rhodoaggretin exists as a $(\alpha\beta)_2$ complex in the native state. We postulate that the di-dimer is stabilized by non-covalent interactions as well as by an intersubunit disulfide bridge between the two αβ-heterodimers. This conclusion is based on the following observations: (a) sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) of the non-reduced rhodoaggretin gave a major 28 and a minor 52 kDa band. (b) Prior treatment of rhodoaggretin with a limited amount of 2-mercaptoethanol (2-ME; 0.1%) resulted in the complete abolishment of the 52 kDa band in SDS–PAGE. (c) Two-dimensional SDS–PAGE in the presence of 3% 2-ME showed that both the 28 and 52 kDa bands gave two bands each with M_rs of 18 (α-subunit) and 15 (β-subunit) kDa. (d) Mass spectrometric analyses showed that purified rhodoaggretin had a M_r of 30 155.39 ± 3.25 Da while its s-pyridylethylated α- and β-subunits had M_rs of 16 535.62 ± 2.98 and 15 209.89 ± 1.61 Da respectively. These molecular weight data suggested the presence of 15 cysteinyl residues in rhodoaggretin as compared to the 14 that are reported for the heterodimeric C-type lectin-like proteins. This extra cysteinyl residue is a candidate for the formation of the intersubunit disulfide bond in the $(\alpha\beta)_2$ complex. (e) Homology structural modeling studies showed that the extra cysteinyl residue can indeed form a disulfide bond that covalently links the two αβ-heterodimers as proposed above.** © **2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: C-type lectin-like protein; Rhodoaggretin; Platelet aggregation; Sequence homology; Molecular modeling; *Calloselasma rhodostoma*

1. Introduction

C-type lectin-like proteins (CLPs) are a group of snake venom proteins that are structurally homologous to the carbohydrate recognition domain of animal C-type lectins [1–10]. Most of these proteins exist as αβ-heterodimers linked by a single interchain disulfide bond, with molecular masses of ~30 kDa. Despite their striking structural similarity, this group of proteins have different effects on blood coagulation and platelet aggregation. Some of these proteins exhibit anticoagulant activities by binding to the coagulant factors X and/or IX [2–4], whereas other CLPs induce varied effects on platelet functions by modulating the interactions between von Willebrand factor (vWF) and platelet glycoprotein Ib (GPIb) [5–9].

Recently, several higher molecular weight multimers of CLPs with different effects on platelet aggregation have also been reported [7,10–13]. These proteins possess native molecular masses of ~50–150 kDa, suggesting that they may represent the dimeric, trimeric or tetrameric forms respectively of the common ~30 kDa disulfide-linked αβ-heterodimers of CLPs. For example, convulxin from the South American rattlesnake *Crotalus durissus terificus* is a 72 kDa protein that may exist as an $(\alpha\beta)_3$ complex [11]. Furthermore, in marked contrast to other CLPs that act on platelets by modulating the interactions between vWF and GPIb, convulxin was reported to induce platelet aggregation via the GPVI collagen receptor [14,15]. Similarly, the 50 kDa alboaggregin A (AL-A) was found to potently induce platelet aggregation of platelet-rich plasma (PRP) [12]. On the other hand, flavocetin (FL)-A and -B from the venom of *Trimeresurus flavoviridis*, with native molecular masses of 149 and 139 kDa respectively, inhibit platelet aggregation at high shear stress [13]. The crystal structure of FL-A has just been published [16]. It is a novel cyclic tetramer $(\alpha\beta)_4$ stabilized by interchain disulfide bonds between heterodimers [16]. It was further concluded that the high affinity of FL-A for the platelet GPIb α-subunit could be explained by a cooperative binding action through the multiple binding sites of the tetramer [16].

In the present study, we propose a model for the three-dimensional structure of rhodoaggretin, a potent platelet aggregation inducer isolated from the venom of *Calloselasma rhodostoma* (Malayan pit viper). It is shown to exist as a novel di-dimeric $(\alpha\beta)_2$ complex in the native state, which is held together by a combination of non-covalent interactions and an intersubunit disulfide linkage. We also provide experimen-

*Corresponding author. Fax: (65)-779-1453.
E-mail address: bchcm@nus.edu.sg (M.C.M. Chung).

Abbreviations: CLPs, C-type lectin-like proteins; vWF, von Willebrand factor; PRP, platelet-rich plasma; *s*-PE, *s*-pyridylethylated; RP-HPLC, reverse-phase high performance liquid chromatography; FPLC, fast protein liquid chromatography; SDS–PAGE, sodium dodecylsulfate–polyacrylamide gel electrophoresis; 2-ME, 2-mercaptoethanol; ESI–MS, electrospray ionization–mass spectrometry

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tal evidence to show that rhodoaggretin is the same protein as aggretin [17,18] and rhodocytin [19].

2. Materials and methods

2.1. Materials

Crude venom of *C. rhodostoma* was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Fast protein liquid chromatography (FPLC) and high performance liquid chromatography (HPLC) columns were from Amersham-Pharmacia Biotech and Vydac respectively, and peptide sequencing chemicals/reagents were from PE-ABD (Foster City, CA, USA). All buffer salts and organic solvents were from standard commercial sources and of the highest quality available.

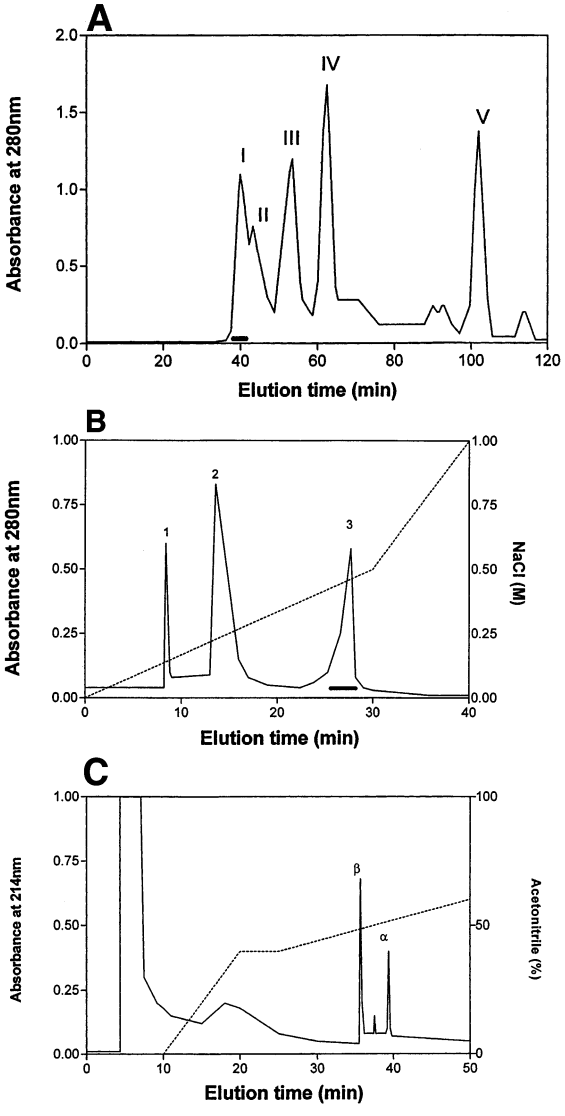

Fig. 1. Purification of rhodoaggretin. A: Gel filtration chromatography of *C. rhodostoma* crude venom on a HiLoad 26/60 Superdex 75 column. Elution was performed at 2 ml/min. B: Anion exchange chromatography on a Mono Q HR 5/5 column. Fractions from the peak I of gel filtration were directly applied to a Mono Q HR 5/5 column that had been equilibrated with 20 mM Tris–HCl buffer, pH 8.2. The protein was eluted at a flow rate of 1 ml/min with a linear gradient of 0–0.5 M NaCl in 20 mM Tris–HCl buffer, pH 8.2 over 30 min. C: Separation of *s*-PE rhodoaggretin α- and β-chains by RP-HPLC on a C_8 Vydac column (4.6×250 mm). Solvent A, 0.1% (v/v) trifluoroacetic acid; B, 0.085% (v/v) trifluoroacetic acid in 70% (v/v) acetonitrile.

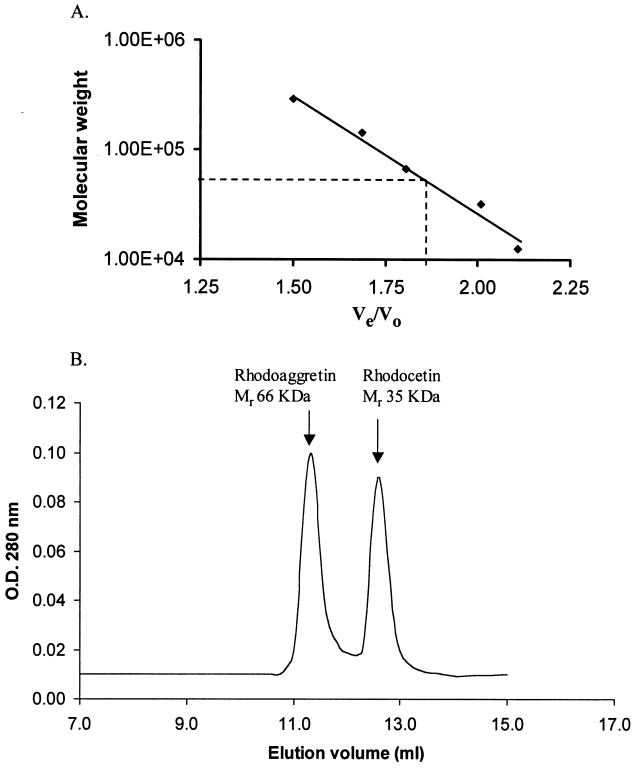

Fig. 2. A: Determination of the native molecular weight of rhodoaggretin. Gel filtration chromatography of standard proteins and purified rhodoaggretin was carried out as described in Section 2. The standard curve was constructed using the molecular weights of standard proteins versus their V_e/V_o values. B: Elution profile of rhodoaggretin and rhodocetin on the same FPLC column.

2.2. Purification of rhodoaggretin

C. rhodostoma crude venom (100 mg) was dissolved in 3.0 ml of 0.1 M ammonium hydrogen carbonate (pH 8.0) and centrifuged at 12 000×*g* for 10 min at 4°C to remove particulate material. The supernatant was then fractionated by a HiLoad Superdex 75 column (2.6×60 cm) equilibrated with the same buffer using a FPLC system. The fractions from peak I (containing proteins of interest based on platelet assays) were pooled and loaded directly onto a Mono Q HR 5/5 column pre-equilibrated with 20 mM Tris–HCl, pH 8.2. Elution was performed with a linear gradient of 0–0.5 M NaCl in 20 mM Tris–HCl buffer, pH 8.2 over 30 min.

2.3. Separation of subunits

Purified rhodoaggretin was reduced and *s*-pyridylethylated (*s*-PE) based on the method of Wang et al. [1]. The α- and β-subunits were subsequently separated by reverse-phase HPLC (RP-HPLC) using a pH stable C_8 Vydac column (4.1×250 mm).

2.4. Molecular weight determination

Molecular weights of the native protein and its subunits were determined by gel filtration, sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and electrospray ionization–mass spectrometry (ESI–MS).

(A) Gel filtration. The native molecular weight of rhodoaggretin was estimated by gel filtration chromatography on a Superose 12 HR 10/30 column using a FPLC system. The column was equilibrated and eluted with 0.1 M ammonium hydrogen carbonate at a flow rate of 0.3 ml/min. Molecular weight standard proteins used included glutamate dehydrogenase (290 000), lactate dehydrogenase (142 000), enolase (67 000), adenylate kinase (32 000) and cytochrome *c* (12 400) (Oriental Yeast Company, Japan).

(B) SDS–PAGE. Non-reducing and reducing (in the presence of 5% 2-mercaptoethanol (2-ME)) SDS–PAGE were performed in 12.5% gel as described previously [20]. For limited reduction of rhodoaggretin,

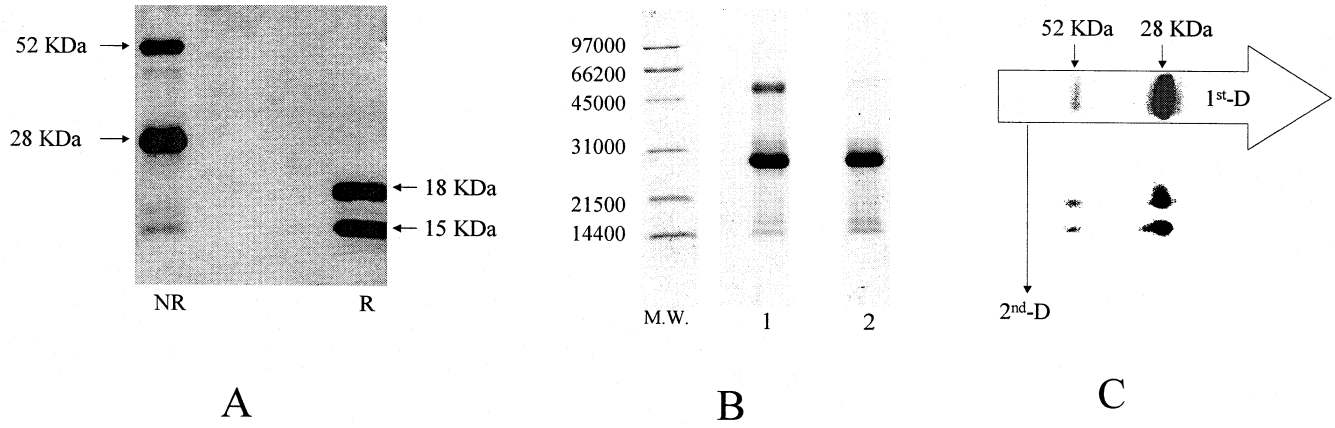

Fig. 3. A: SDS–PAGE of rhodoaggretin in the presence (R) and absence (NR) of 2-ME. B: SDS–PAGE in the absence (lane 1) and presence of 0.1% 2-ME (lane 2). M.W., molecular weight markers. C: 2D SDS–PAGE of rhodoaggretin. The first dimension was carried out in the absence of 2-ME.

different final concentrations (0.03, 0.1, and 0.3%) of 2-ME were added to treat the samples before electrophoresis.

(C) Two-dimensional (2D) SDS–PAGE. One-dimensional non-reducing SDS–PAGE was performed as in (B). After electrophoresis, one lane was excised from the unstained gel, and incubated in SDS sample buffer containing 3% 2-ME for 0.5 h. This treated gel strip was then transferred to the top of a second 12.5% SDS gel before electrophoresis in the second dimension.

(D) ESI–MS. MS was carried out using a Perkin-Elmer Sciex API 300 LC/MS/MS system, a triple-stage quadruple instrument equipped with an ionspray interface. The ionspray voltage was set to 4000 V, orifice voltage at 75 V, and the interface temperature at 60°C. Nitrogen was used as a curtain gas with a flow rate of 0.6 l/min, and as a nebulizer gas at 30 psi. A Shimadzu LC-10AD series pump system was used for solvent delivery.

2.5. Determination of the isoelectric point

The isoelectric point of rhodoaggretin was determined using agarose IEF in a pH range of 3–10 (Pharmalyte) based on the manufacturer's protocol.

2.6. Determination of N-terminal amino acid sequence

The N-terminal amino acid sequences of the α- and β-subunits of *s*-PE rhodoaggretin were determined by automated Edman degradation using an Applied Biosystems 477A pulsed liquid-phase sequencer equipped with an on-line PTH amino acid analyzer (120A).

2.7. Platelet aggregation

Blood drawn from the central arteries of rabbit ears was anticoagulated with 0.11 M tri-sodium citrate (1:9, v/v). PRP was obtained by centrifugation of the blood for 20 min at $375 \times g$ and 20°C. Washed platelets were prepared as described previously [21]. Platelet aggregation in whole blood was measured by the impedance method [22], using a Chronolog Model 500-CA whole blood aggregometer (Chronolog, Havertown, PA, USA), under continuous stirring at 1000 rpm. Platelet aggregation in PRP and washed platelets, on the other hand, were monitored by light transmission [23].

2.8. Peptide mapping

The *s*-PE α- and β-subunits of rhodoaggretin were digested with endoproteinase Lys-C and the peptides fractionated by RP-HPLC as described earlier [1]. Selected peptides were analyzed by ESI–MS, as detailed above.

2.9. Homology modeling

Homology structural modeling of aggretin (rhodoaggretin) was carried out using the structure of coagulation factor (IX/X) binding protein [24] as the target template. The LOOK modeling package [25] was used to first construct a monomer of each of the molecules. The 'model homolog' option was used so that the entire molecule including loops could be modeled. This option also allowed modeling of the loops using segment match modeling, which uses a database of

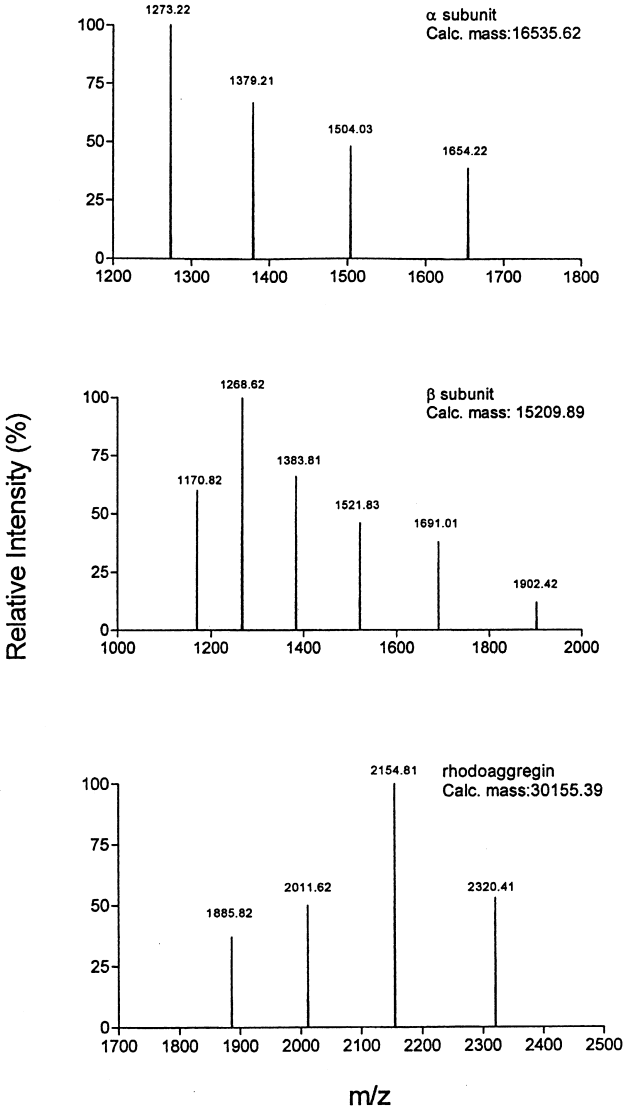

Fig. 4. ESI–MS spectra of the *s*-PE α- and β-subunits of rhodoaggretin and unmodified rhodoaggretin.

highly refined protein structures to find similar fragments to the target structure that is being modeled and subsequently this model is subjected to energy minimization [26]. The resulting structure was subsequently loaded into the modeling package SYBYL 6.6 (Tripos Inc., MO, USA) and one or two copies of the molecule were made. Multimers were then created manually by moving the molecules so that free cysteinyls would be positioned close to each other allowing thus for disulfide formation.

3. Results

3.1. Purification of rhodoaggretin

The crude venom of *C. rhodostoma* was separated into five main fractions by Superdex 75 gel filtration chromatography (Fig. 1A). Fraction I, which was found to induce platelet aggregation potently in rabbit and human whole blood, PRP and washed platelets, was thus subjected to further fractionation on a Mono Q column. This purification step resulted in three fractions (Fig. 1B) of which only fraction 3 exhibited marked aggregatory activity towards platelets. This fraction was designated as rhodoaggretin. It is an acidic protein with an isoelectric point (p*I*) of 3.45.

α subunit

	1				5				10				15				20			
Rhodoaggretin	**G**	**L**	**E**	**D**	**C**	**D**	**F**	**G**	**W**	**S**	**P**	**Y**	**D**	**Q**	**H**	**C**	**Y**	**Q**	**A**	**F**	**N**	**E**
Rhodocytin	G	L	E	D	C	D	F	G	W	S	P	Y	D	Q	H	C	Y	Q	A	F	N	E
Aggretin	G	L	E	D	C	D	F	G	W	S	P	Y	D	Q	H	C	Y	Q	A	F	N	E
Convulxin	G	L	-	H	C	P	S	D	W	Y	Y	Y	D	Q	H	C	Y	R	I	F	N	E
Flavocetin A		D	F	D	C	I	P	G	W	S	A	Y	D	R	Y	C	Y	Q	A	F	S	K
Rhodocetin				D	C	P	D	G	W	S	S	T	K	S	Y	C	Y	R	P	F	K	E
Botrocetin				D	C	P	S	G	W	S	S	Y	E	G	N	C	Y	K	F	F	Q	Q
Alboaggregin-B				D	C	P	S	D	W	S	S	F	K	Q	Y	C	Y	Q	I	F	K	Q
Echicetin		D	Q	D	C	L	S	G	W	S	F	Y	E	G	H	C	Y	Q	L	F	R	L
ECLV IX/X-bp				D	C	L	P	G	W	S	S	H	E	G	H	C	Y	K	V	F	N	E
Habu IX/X-bp				D	C	L	S	G	W	S	S	Y	E	G	H	C	Y	K	A	F	E	K
Habu IX-bp				D	C	P	S	G	W	S	S	Y	E	G	H	C	Y	K	P	F	K	L
Jararaca GPIb-bp	D	T	P	F	E	C	P	S	D	W	S	T	H	R	Q	Y	C	Y	K	F	F	Q	Q

β subunit

	1				5				10				15				20			
Rhodoaggretin			**D**	**C**	**P**	**S**	**G**	**W**	**S**	**S**	**Y**	**E**	**G**	**H**	**C**	**Y**	**K**	**P**	**F**	**N**	**E**
Rhodocytin			D	C	P	S	G	W	S	S	Y	E	G	H	C	Y	K	P	F	N	E
Aggretin			D	C	P	S	G	W	S	S	Y	E	G	H	C	Y	K	P	F	N	E
Convulxin	G	F	C	C	P	S	H	W	S	S	Y	D	R	Y	C	Y	K	V	F	K	Q
Flavocetin A	G	F	C	C	P	L	G	W	S	S	Y	D	E	H	C	Y	Q	V	F	Q	Q
Rhodocetin	D	F	R	C	P	T	T	W	S	A	S	K	L	Y	C	Y	K	P	F	K	E
Botrocetin			D	C	P	P	D	W	S	S	Y	E	G	H	C	Y	R	F	F	K	E
Alboaggregin-B			D	C	P	S	D	W	S	S	Y	D	L	Y	C	Y	R	V	F	Q	E
Echicetin			N	C	L	P	D	W	S	V	Y	E	G	Y	C	Y	K	V	F	K	E
ECLV IX/X-bp			D	C	S	S	G	W	T	A	Y	G	K	H	C	Y	K	V	F	D	E
Habu IX/X-bp			D	C	P	S	D	W	S	S	Y	E	G	H	C	Y	K	P	F	S	E
Habu IX-bp			D	C	P	S	D	W	S	S	Y	E	G	H	C	Y	K	P	F	S	E
Jararaca GPIb-bp			D	C	P	S	D	W	S	P	Y	G	G	H	C	Y	K	L	F	K	Q

Fig. 5. Comparison of the N-terminal amino acid sequences of rhodoaggretin with those of CLPs from other snake venoms. The N-terminal amino acid sequences of the α- and β-subunits of rhodoaggregin are compared with the CLPs isolated from other snake venoms, including rhodocytin [19], aggretin [18], convulxin [11], FL-A [16], rhodocetin [1], botrocetin [27], alboaggregin B [28], echicetin [29], ECLV IX/X-bp [2], habu factor IX/X-bp [3], habu factor IX-bp [4] and jararaca GPIb-bp [30].

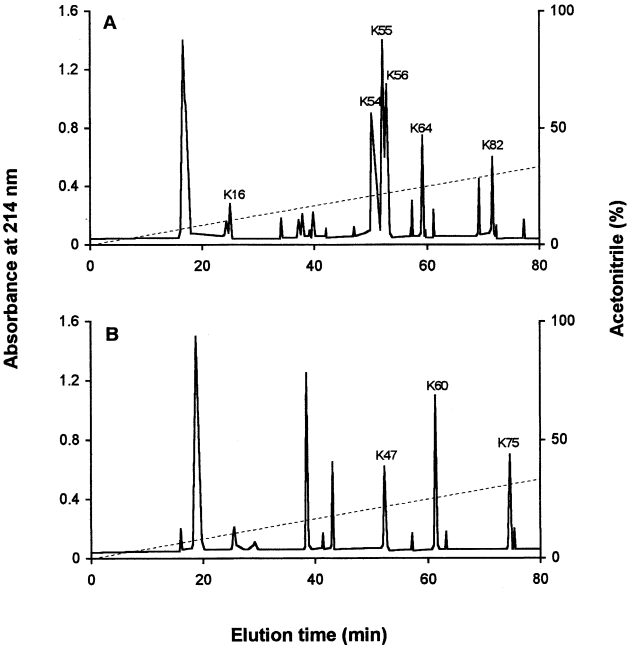

Fig. 6. Peptide mapping of the *s*-PE (A) α- and (B) β-subunits of rhodoaggretin after proteolytic digestion by endoproteinase Lys-C.

3.2. Determination of the molecular weight of rhodoaggretin

The native molecular weight of rhodoaggretin was determined to be 66 000 by gel filtration chromatography on a Superose 12 column (Fig. 2A). In a separate experiment, the relative elution volumes for rhodoaggretin and rhodocetin are compared (Fig. 2B). On reducing SDS–PAGE, rhodoaggretin exhibited two bands with molecular masses of 18 and 15 kDa (designated as α- and β-subunits respectively) (Fig. 3A). Under non-reducing conditions, however, two distinctive bands with apparent molecular masses of 28 kDa and 52 kDa were observed (Fig. 3A). At a concentration of 0.1% 2-ME, the 52 kDa band was abolished, and only the 28 kDa band remained as the predominant component (Fig. 3B). This suggested that the 52 kDa protein is an interdisulfide-linked dimer of the 28 kDa protein. This conclusion is supported by the result of the 2D SDS–PAGE experiment which showed

Table 1
Mass spectrometric analysis of α- and β-subunits and purified peptides from endoproteinase Lys-C digestion of rhodoaggretin

Peptides	Residue no.[a]	Calculated[b]	Observed[c]
α-Subunit			
Intact	1–136	16 531.83	16 534.43 ± 2.98
K16	112–117	721.43	721.70 ± 0.16
K54	118–132	2130.94	2 130.56 ± 0.18
K55	1–24	3 090.28	3 090.55 ± 0.98
K56	80–104	2 947.30	2 947.59 ± 0.91
K64	62–79	2 135.04	2 134.88 ± 0.37
K82	32–61	3 412.64	3 413.13 ± 0.74
β-Subunit			
Intact	1–23	15 209.19	15 209.89 ± 1.61
K47	36–53	2 030.00	2 030.34 ± 0.26
K60	89–120	4 159.81	4 160.13 ± 0.18
K75	61–87	3 424.63	3 424.51 ± 1.75

[a]Based on aggretin sequence.
[b]Molecular masses calculated from the amino acid sequences of the denoted peptides of aggretin.
[c]Molecular masses calculated from multiple charged signals observed.

that both the 52 and 28 kDa bands gave rise to 18 and 15 kDa bands in the presence of excess (3%) 2-ME (Fig. 3C).

Purified rhodoaggretin, which eluted as a single peak in RP-HPLC, gave a molecular mass of 30 155.39 ± 3.25 Da when analyzed by ESI–MS (Fig. 4). The α- and β-subunits, after reduction and pyridylethylation, were separated by RP-HPLC (Fig. 1C), and their molecular masses as determined by ESI–MS were 16 535.62 ± 2.98 and 15 209.89 ± 1.61 Da respectively (Fig. 4).

3.3. N-terminal amino acid sequence analysis

The N-terminal amino acid sequences of the α- and β-subunits of rhodoaggretin were determined by sequencing the individual reduced and *s*-PE subunits (Fig. 5). A high degree

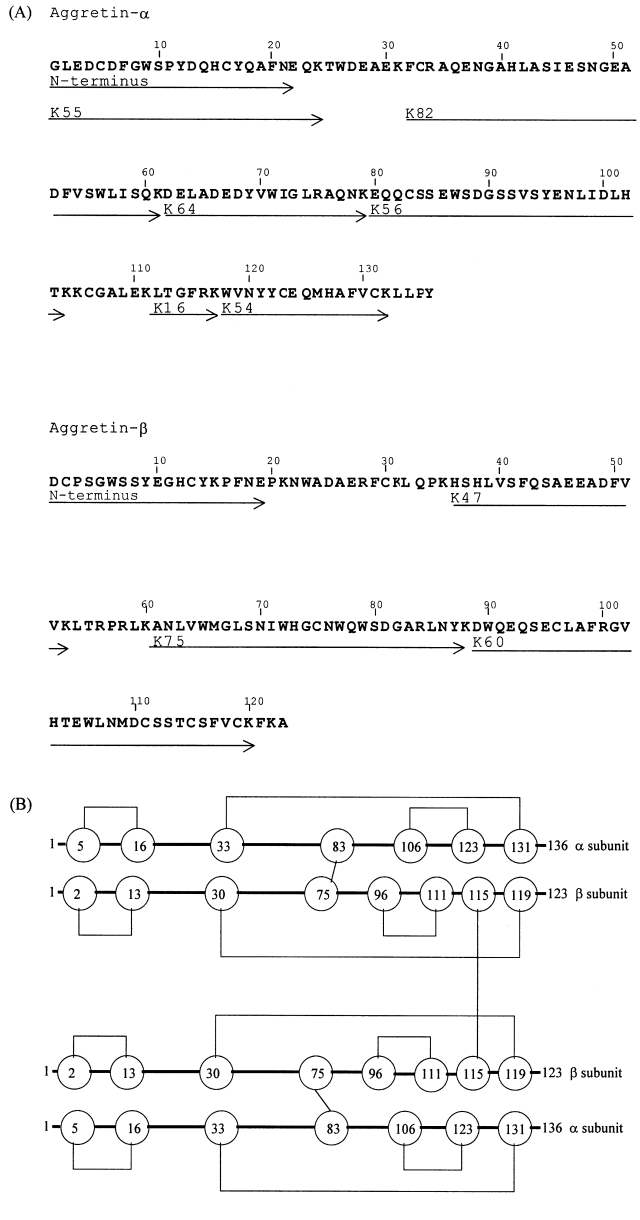

Fig. 7. A: The sequences of the Lys-C peptides of rhodoaggretin (—) that matched the corresponding peptide sequences of aggretin [18], based on the MS data (Table 1). B: The location of the intra- and intermolecular disulfide bridges of each rhodoaggretin (aggretin) heterodimer, and the proposed disulfide bond that is formed by Cys115–Cys115 of the two β-chains of the di-dimer.

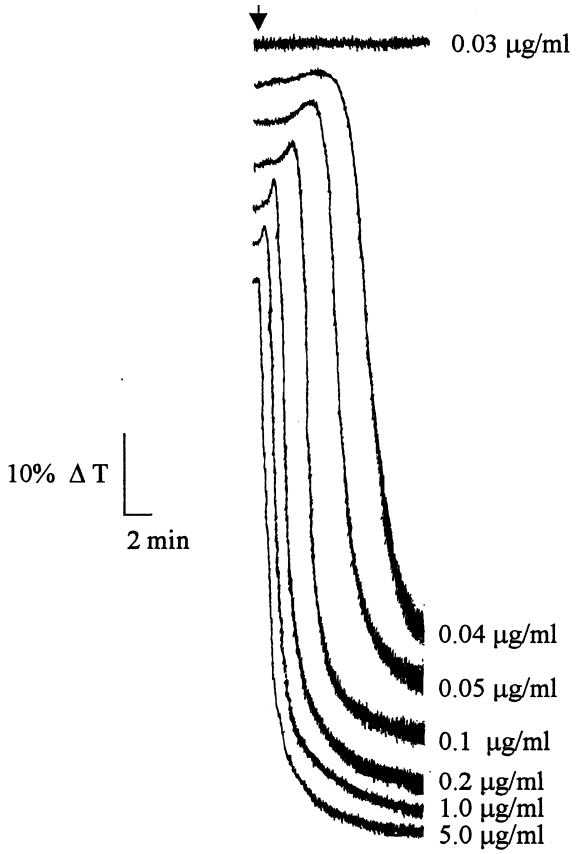

Fig. 8. Platelet aggregation induced by various concentrations of rhodoaggretin. Various concentrations of rhodoaggretin (0.03–5.0 μg/ml) were added to washed rabbit platelet suspension to induce platelet aggregation. The arrows mark the addition of rhodoaggretin. ΔT denotes the change in light transmission.

of sequence identity was observed between the two subunits. When aligned with those of other known members of the CLP superfamily from snake venoms (Fig. 5), the α- and β-subunits of rhodoaggretin showed identical N-terminal sequences with rhodocytin [19] and aggretin [18] and a relatively high degree of sequence identity with rhodocetin [1], convulxin [10,11], FL-A [16], botrocetin [27], alboaggregin B [28], echicetin [29], ECLV IX/X-bp [2], habu IX/X-bp [3], habu IX-bp [4] and jararaca GPIb-bp [30].

3.4. Peptide mapping studies

The peptide maps for the *s*-PE α- and β-subunits of rhodoaggretin are shown in Fig. 6A,B respectively. The molecular masses for the selected peptides as determined by ESI–MS are summarized in Table 1. These peptide masses were compared with the calculated masses for the corresponding peptides of aggretin whose complete sequence was published recently [18]. Fig. 7 also showed the alignment of these peptides with the complete amino acid sequences of the α- and β-subunits of aggretin.

3.5. Effect of rhodoaggretin on platelet aggregation

Rhodoaggretin itself could induce platelet aggregation in rabbit and human whole blood, PRP and washed platelets with a lag period in an all-or-none manner (Fig. 8). For example, at a concentration of 0.03 μg/ml, it did not induce any aggregation, but at 0.04 μg/ml, it resulted in maximal aggre-

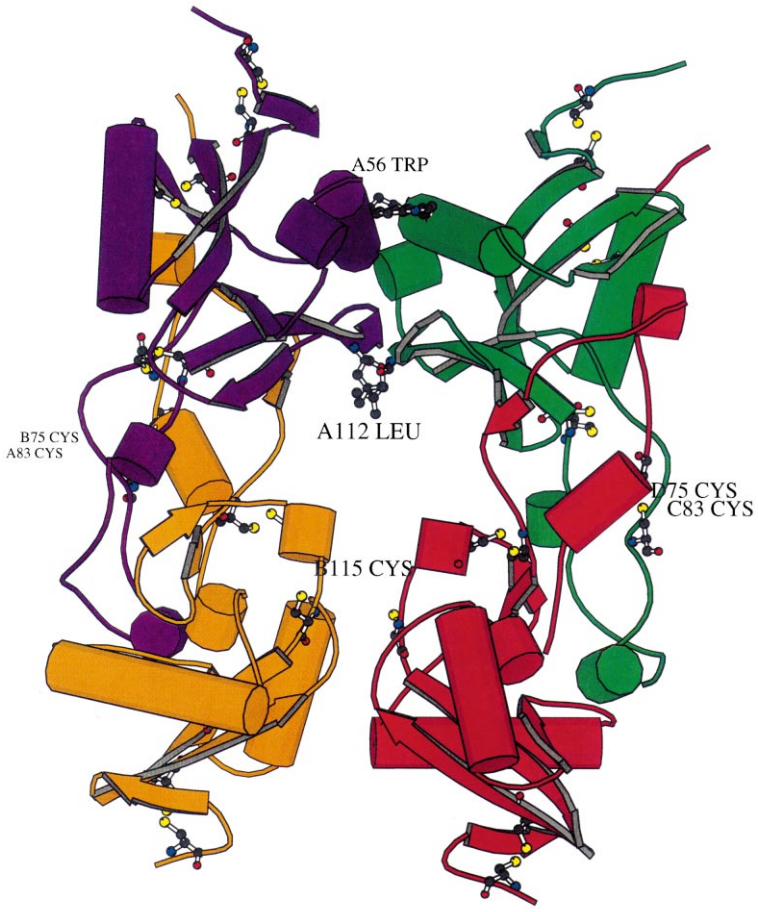

Fig. 9. A rendering of putative (rhodo)aggretin dimer using Molscript [35]. α-Subunits are colored in purple and green while their corresponding β-subunits are colored in orange and crimson respectively. Residues Cys75 of the β-chain and Cys83 of the α-chain forming the interdisulfide bond within the same heterodimer are labeled as A83–B75 and C83–D75 for the two independent dimers. Residues Cysβ115, Leuα112, and Trpα56 are also labeled and show how they can stabilize the molecule by interacting with their dimer-related residues.

gation (Fig. 8). Interestingly, the initial latent period of aggregation was a direct function of protein concentration.

3.6. Molecular modeling studies

The aggretin (rhodoaggretin) model (Fig. 9) showed that it contained three intracaternary disulfide bonds within each of the α- (Cys5–16, Cys33–131, Cys106–123) and β- (Cys2–13, Cys30–119, Cys96–111) chains. Residues Cys75 from the β-chain and Cys83 from the α-chain form the interdisulfide bond between these subunits. Most interestingly, the extra cysteine residue (Cys115) from the β-chain of each aggretin heterodimer can form a disulfide bond with the β-chain of another heterodimer, which thus stabilizes the 52 kDa dimer. In addition, residues Leu112 and Trp56 from two dimer-related α-chains help to bridge the intermolecular contact region with a hydrophobic area (Fig. 9).

4. Discussion

Based on the observations made in this study, we suggest that rhodoaggretin, rhodocytin and aggretin are the same protein found in the venom of *C. rhodostoma*. With the recent publication of the complete sequence of aggretin [18] we were able to confirm that rhodoaggretin is indeed the same protein as aggretin by peptide mapping, and mass spectrometric analyses. It was not clear why under non-reducing conditions aggretin and rhodocytin were not shown earlier to contain any bands with $M_r > 50\,000$ [17,19]. In a recent paper, however, Navdaev et al. [31] reported that their aggretin preparation did contain 60 and 28 kDa bands under non-reducing SDS–PAGE.

Several high molecular weight potent platelet agonists/antagonists isolated from snake venoms have been identified as CLP superfamily members [7,10–13,32]. These proteins usually possess molecular masses in excess of 50 kDa under non-reducing conditions of SDS–PAGE, but upon reduction gave rise to heterodimeric 12–15 kDa subunits with N-terminal amino acid sequences that are highly homologous to the CLPs. These proteins are also usually found in venoms that already contained the well-characterized heterodimeric CLPs. Hitherto, the quaternary structures of these multimeric CLPs are not known, but with the recent publication of the crystal structures of FL-A [16], factor IX/X-bp [24], factor IX-bp [33], and botrocetin [34] it has become possible to predict the structures of some of these proteins based on molecular homology modeling studies. FL-A, with a M_r of 149 kDa, has been shown to be a novel tetramer $(\alpha\beta)_4$ made up of four αβ-heterodimers related by a crystallographic four-fold symmetry [16]. The tetramerization is mediated by an interchain disulfide bridge between cysteinyl residues (Cysα135) at the C-ter-

minus of the α-subunit and at the N-terminus of the β-subunit (Cysβ3) in the neighboring αβ-heterodimer [16]. Therefore, the additional cysteinyl residues in the α- and β-subunits of each αβ-heterodimer seem to hold the key to the formation of this type of multimeric structure.

Our results here suggest that rhodoaggretin (aggretin, rhodocytin), which has a native M_r of 60 kDa, is a dimer of the αβ-heterodimer. The stabilization of the dimer seems to involve both an interdisulfide bond and non-covalent interactions between the αβ-heterodimers. Firstly, we established that there was an extra cysteinyl residue in rhodoaggretin based on the MS results of the *s*-PE α- and β-subunits and the unmodified αβ-heterodimer. We found there were 15 instead of the expected 14 cysteinyl residues that were reported for all known αβ-heterodimeric CLPs [2–9], except rhodocetin [1]. This finding was subsequently confirmed by the publication of the complete sequence of aggretin, in which an additional cysteinyl residue was located at position 115 in the β-chain [18]. It could thus be postulated that Cysβ115 is a possible site for the dimerization of the αβ-heterodimer via disulfide formation between the β-chains of each heterodimer. Our SDS–PAGE results under non-reducing conditions supported this suggestion since the minor 52 kDa band could be removed completely by 0.1% of 2-ME (Fig. 3B). However, the major stabilizing force in the dimerization of the αβ-heterodimer is non-covalent in nature. Our structural modeling studies based on the published structure of factor IX/X-bp were in full accord with this conclusion (Fig. 9). This conclusion is to be contrasted with the result for AL-A, in which under non-reducing SDS–PAGE, only a 52 kDa band was evident [7,12,32], suggesting dimerization was mainly a result of interdisulfide bond formation between the subunits. However, unlike rhodoaggretin, AL-A was shown to consist of four different polypeptide chains of 111–132 amino acids in length, and Kowalska et al. [32] had proposed how the individual chains are stabilized by inter- and intradisulfide bonds. However, there was no structural evidence to support their suggestion [32].

In summary, we have isolated and characterized a di-dimeric CLP with a novel degree of oligomerization. In contrast to of FL-A [16] and CVX [11], where the tetrameric and trimeric structures are held together in a 'head-to-tail' configuration by disulfide bonds respectively, the rhodoaggretin dimer is stabilized mainly by non-covalent forces and a disulfide bond between the two β-subunits probably via a 'side-to-side' interaction. Since the extra cysteinyl residue (Cysβ115) is not the C-terminal amino acid, and hence not so flexible, we suggest that the disulfide bond formed between the β-subunits from each αβ-heterodimer is most probably a random process, thus resulting only in a minor contribution to the stabilization of the dimeric structure of rhodoaggretin.

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