Amino acid sequence of the α subunit and computer modelling of the α and β subunits of echicetin from the venom of *Echis carinatus* (saw-scaled viper)

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Echicetin, a heterodimeric protein from the venom of *Echis carinatus*, binds to platelet glycoprotein Ib (GPIb) and so inhibits platelet aggregation or agglutination induced by various platelet agonists acting via GPIb. The amino acid sequence of the β subunit of echicetin has been reported and found to belong to the recently identified snake venom subclass of the C-type lectin protein family. Echicetin α and β subunits were purified. N-terminal sequence analysis provided direct evidence that the protein purified was echicetin. The paper presents the complete

amino acid sequence of the α subunit and computer models of the α and β subunits. The sequence of α echicetin is highly similar to the α and β chains of various heterodimeric and homodimeric C-type lectins. Neither of the fully reduced and alkylated α or β subunits of echicetin inhibited the platelet agglutination induced by von Willebrand factor–ristocetin or α -thrombin. Earlier reports about the inhibitory activity of reduced and alkylated echicetin β subunit might have been due to partial reduction of the protein.

INTRODUCTION

Echicetin, a heterodimeric protein isolated from the venom of *Echis carinatus* (saw-scaled viper), is composed of a 16 kDa α subunit and a 14 kDa β subunit [1]. Echicetin binds to platelet glycoprotein Ib (GPIb) and so inhibits platelet aggregation or agglutination induced by various platelet agonists acting via GPIb, such as human von Willebrand factor (vWf)–ristocetin/botrocetin, bovine vWf and alboaggregins [1]. Platelet aggregation and secretion induced by low concentrations of thrombin are also inhibited by echicetin [2,3]. The amino acid sequence of the β subunit of echicetin has been published recently. Echicetin β subunit is highly similar to the β chains of botrocetin [4], factor IX/X-binding protein [5], alboaggregin-B [6] and homodimeric C-type lectins [7,8]. Reduced and alkylated echicetin β subunit has been reported to retain a high percentage of the inhibitory activity of echicetin, and on the basis of this the biological activity of echicetin has been proposed to be due to its β subunit [2].

Here we report the complete amino acid sequence of the α subunit and a computer model of both α and β subunits of echicetin. In our hands neither reduced and alkylated α or β subunits of echicetin inhibited thrombin-induced or vWf–ristocetin-induced platelet aggregation, which is contrary to the findings of Peng et al. [2]. A possible source of this contradiction, as well as the location of the binding site for GPIb within the heterodimer, is discussed.

MATERIALS AND METHODS

Purification of echicetin

Echicetin was purified from freeze-dried *E. carinatus sochureki* venom (Latoxan, Rosans, France) as described by Peng et al. [1] with the modification that ion-exchange chromatography was

used as the first step and reverse-phase HPLC as the second step of the purification. Purified echicetin was freeze-dried and stored at 4 °C.

Purification of α and β subunits of echicetin

To 1 vol. of 1 mg/ml echicetin in 6 M guanidinium chloride/0.1 M Tris (pH 8.0) was added 1/20 vol. of 440 mM dithiothreitol and the mixture was incubated at 45 °C for 30 min; 1/20 vol. of 1 M 4-vinylpyridine was added and after 1 h of incubation at room temperature the sample was diluted with 2 vol. of 0.2 % trifluoroacetic acid. The reduced S-pyridylethylated echicetin subunits were isolated free of reagents by reverse-phase HPLC.

Sequencing of echicetin α

Sequence analysis was performed on an Applied Biosystems model 477A pulsed-liquid-phase protein sequencer with a model 120A on-line phenylthiohydantoin amino acid analyser. N-terminal sequencing of echicetin α yielded 30 residues from the N-terminus. Aliquots of 20 μ g of echicetin α were used for enzymic and CNBr digestions. Endoproteinase Asp-N (Boehringer Mannheim; 2 %, w/v) was added to the protein dissolved in 100 μ l of 100 mM Tris/HCl, pH 8.0, and incubated at 37 °C for 18 h. Endoproteinase Glu-C (*Staphylococcus aureus* V8; Boehringer Mannheim; 3.5 %, w/v) was added to the protein dissolved in 100 μ l of 25 mM NH_4HCO_3 , pH 7.8, and incubated at 37 °C for 18 h. For pepsin digestion, the protein was dissolved in 100 μ l of 3.5 % (v/v) formic acid; 2 μ g of pepsin (Sigma; 10 %, w/v) was added and the solution was incubated for 18 h at room temperature. For CNBr cleavage, the protein was dissolved in 100 μ l of 70 % (v/v) formic acid containing 10 mg/ml CNBr and incubated for 18 h in the dark at room temperature. The peptides generated by proteolytic and CNBr digestions were separated by

Abbreviations used: GPIb, glycoprotein Ib; MBP, mannose-binding protein; vWf, von Willebrand factor.

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The nucleotide sequence data reported here will appear in DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession number P51741.

reverse-phase HPLC (Hewlett Packard HP1090), with a Brownlee C₁₈ column (220 mm × 2.1 mm). The peptides were eluted with an acetonitrile gradient (in 0.1 % trifluoroacetic acid), from 0 % to 50 % over 60 min, followed by 50–65 % over 5 min. The elution was monitored with a diode-array detector at 214 and 280 nm; all of the peaks, collected manually, were sequenced.

Amino acid determination

Echicetin α (1 nmol) was hydrolysed in the gas phase under vacuum in a nitrogen atmosphere at 112 °C for 24 h in 6 M HCl containing 1 mg/ml phenol; and the resultant amino acids were measured with the Waters AccQ.Tag chemistry and a Hewlett Packard HP1090 liquid chromatograph.

Electrospray MS

Electrospray mass spectra were obtained on a Trio 2/3000 ESI instrument (VG Biotech). Samples from reverse-phase HPLC (trifluoroacetic acid/acetonitrile system) were injected directly into the solvent stream of methanol/water/acetic acid (49.5:49.5:1, by vol.), or were dried, then dissolved in the latter solvent system and were infused at a rate of 2 μ l/min. Spectra were collected over the m/z range 700–1900 and co-added by repetitive scanning until an acceptable signal-to-noise ratio was obtained (30 scans). Lab-Base software was used to deconvolute the spectra. Masses were obtained nine times, giving a mean $\pm$ S.D. value of 16540.49 $\pm$ 0.43 Da, which was then corrected for the cysteine derivatization to give a mass of 15805 Da for the echicetin α subunit, compared with the calculated mass of 15803 Da.

Computer analysis of the sequence results

Computer analysis of the sequence data was performed on a VAX/VMS system using the suite of programs from the University of Wisconsin Genetics Computer Group. Initial searches were performed with the Dayhof similarity against the Swissprot databases. Percentage similarity values between α echicetin and α and β chains of various C-type lectins were determined with the program BESTFIT, with a gap-creating penalty of 3.0 and a gap-extension penalty of 0.1.

Protein determination

Protein determination was performed by the bicinchoninic acid protein assay (Pierce Chemical Co, Rockford, IL, U.S.A.) with BSA as a standard.

SDS/PAGE/silver staining

SDS/PAGE was performed by the method of Laemmli [9] and the gels were silver stained by the method of Morrissey [10].

Preparation of washed platelets and platelet aggregation

Human platelets were isolated from buffy coats, less than 20 h after blood collection, obtained from the Central Laboratory of the Swiss Red Cross Blood Transfusion Service. To one buffy coat was added 30 ml of 100 mM citrate, pH 6.5. Platelet-rich plasma and the platelet pellet were isolated by successive centrifugation steps. Platelets were resuspended in a small volume of 113 mM NaCl/4.3 mM K₂HPO₄/4.3 mM Na₂HPO₄/24.4 mM NaH₂PO₄/5.5 mM glucose (pH 6.5) (buffer A). Iloprost (a gift from Schering AG, Zürich, Switzerland) (10 ml, 0.5 mg/ml) was added, the suspension was diluted to 40 ml with buffer A and centrifuged at 250 g for 5 min. The platelet-rich supernatant was

centrifuged at 1000 g for 10 min and platelets were washed twice with buffer A. Washed platelets were resuspended in 20 mM Hepes/140 mM NaCl/4 mM KCl/5.5 mM glucose (pH 7.4) (buffer B) and the platelet count was adjusted to 6×10^8 to 10^9 platelets/ml by dilution with buffer B. Samples were kept at room temperature until use for aggregation studies. Platelet aggregation was monitored by light transmission [11] with a Lumitec aggregometer with continuous stirring at 1300 rev./min at 37 °C. Platelets were preincubated in buffer containing 2 mM CaCl₂ at 37 °C for 2 min before starting the measurement by adding the samples for analysis.

Computer modelling

The three-dimensional structures of echicetin α and β subunits were modelled with comparative protein modelling methods as implemented in the automated protein modelling server Swiss-Model [12,13]. As both echicetin α and β show a clear similarity to other snake venom C-type lectins, and because both sequences contain the corresponding PROSITE pattern (PS00615), we hypothesized that modelling of these proteins based on the C-type lectin fold could be feasible. Both echicetin α and β sequences were used to search a database of sequences derived from known protein structures by using BLASTP [14]. Only very weak similarities to sequences of known structure were found, the best being for C-type lectins. To confirm the latter fold as suitable for the modelling procedure, we used the programme THREADER [15], which predicted both echicetin subunits to have the same fold as mannose-binding protein (MBP) (Brookhaven Protein Data Bank entry code 2MSB) [16,17] with z scores of -3.9 . A sequence alignment, maximizing the sequence identity and respecting the secondary structure elements of MBP, was generated by a combination of a dynamic programming algorithm as implemented in SIM [18] and manual corrections, which always yield the best results in borderline cases. Both C-terminal C-type lectin PROSITE and cysteine-bonding patterns were used as guidelines in this process. The resulting sequence alignment shows that only 10 residues are conserved in both echicetin subunits and MBP, and seven more are identical between either echicetin subunit and MBP. The overall sequence identity level with MBP is thus between 12 % and 13 %. Such low levels of sequence identity are not unprecedented between proteins with very similar tertiary structures. Indeed, the members of the lipocalin family (e.g. plasma retinol-binding protein, bilin-binding protein, apolipoprotein D) share only 12 identities for a sequence overlap of 180 residues, whereas their structures differ by only 2 Å relative mean square deviation [19]. Because protein models, even of relatively low precision, yield important topological information, we built models for both echicetin subunit sequences based on MBP. The final model structures were inspected for sequence to structure fitness using both three-dimensional–one-dimensional profiles [20] and ProsaII [21]. Three-dimensional–one-dimensional scores of 0.41 (0.50 for PDB entry 2MSB) and ProsaII z scores of -6.5 (-7.8 for PDB entry 2MSB) were obtained for both models. In addition, no markedly inconsistent regions were detected. This demonstrates that both echicetin subunit sequences most certainly adopt the C-type lectin fold, and that the sequence alignment described above, as well as the resulting models, is sound.

RESULTS AND DISCUSSION

The fractionation of reduced S-pyridylethylated echicetin subunits is shown in Figure 1. Peaks in the 3–10 min interval represent non-protein peaks originating from the reagents used

Figure 1 Fractionation of reduced and S-pyridylethylated echicetin on a reverse-phase HPLC column

The line with peaks 1 and 2 shows $10^3 A_{216}$ (mAU); the other line is the percentage of solvent B [80% (v/v) acetonitrile in 0.08% (v/v) trifluoroacetic acid]. Peak 1, echicetin α subunit; peak 2, echicetin β subunit; open arrow, position of non-reduced echicetin. Note that peaks in the 3–10 min interval originated from the reagents used for reduction and S-pyridylethylation.

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1      10      20      30      40      50      60      70
DQDCLSGWSEFYEGHCYQLFRLKTWDEAEKYCNQWDGGHVLVSIESNAKAEFVAQLISRLPKSAIEDRVWI
71      80      90      100     110     120     130
GLRDRSKREQCQGLHWLTDNSFVHYEHVVPPTKCFVLERQTEFRKWIAVNCEFKFPPVCKAKIPR

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Figure 2 Amino acid sequence of echicetin α subunit

for reduction and S-pyridylethylation. No non-reduced echicetin could be detected (open arrow). Fractions 1 and 2 revealed homogeneous single-chain proteins with apparent molecular masses of 16 and 14 kDa respectively, analysed by SDS/PAGE/silver staining (results not shown). N-terminal sequence analysis of fraction 2 resulted in a 30-residue sequence identical with the N-terminal part of echicetin β subunit reported recently [2]. This provided direct evidence that the protein purified was echicetin. The complete amino acid sequence of echicetin α subunit, fraction 1, was determined (Figure 2). The results of amino acid analysis of echicetin α subunit (results not shown) supported the sequence depicted in Figure 2. The α subunit of echicetin contains 133 amino acid residues, including seven of cysteine. A good correlation was found between the molecular mass calculated from the amino acid sequence, 15803 Da, and that measured by electrospray MS, 15805 Da. The α subunit of echicetin shows a high degree of similarity to other hetero- and homo-dimeric snake venom C-type lectins, especially to the α chains of *Trimeresurus flavoviridis* factor IX-binding protein [22] (52.8% similarity), *Trimeresurus albolabris* alboaggregin B [6] (51.2%), *T. flavoviridis* factor IX/X-binding protein [5] (50.4%), *E. carinatus leucogaster* factor IX/X-binding protein [23] (50.0%), *Bothrops jararaca* GPIIb-binding protein [24] (49.6%) and *B. jararaca* botrocetin [4] (49.2%). The α and β subunits of echicetin show 35.5% similarity to each other.

The IC_{50} concentrations of non-reduced echicetin for inhibition of bovine vWf-induced and α -thrombin-induced platelet agglutination/aggregation have been found to be 0.015 and 0.042 mM respectively [2]. Although we found similar values for non-reduced echicetin by analysing its inhibitory effect on vWf-ristocetin-induced platelet agglutination and thrombin-induced aggregation, no inhibitory activity was found with either the reduced α subunit or the reduced β subunit of echicetin. This is contrary to the results of Peng et al. [2]. However, these authors

Figure 3 Computer model of the echicetin α subunit based on the structure of MBP

The truncation of the Ca^{2+} -binding loop in echicetins compared with MBP is indicated.

found that: (1) after reduction and alkylation of echicetin, the largest peak eluted from the reverse-phase HPLC column was non-reduced echicetin (fraction 3 in Figure 1 in [2]); (2) both echicetin α and β subunits showed double peaks in the chromatogram (fractions 1, 2 and 4, 5 in Figure 1 in [2]); (3) both echicetin α and β subunits showed two bands with different migrations as analysed by SDS/PAGE (Figure 2 in [2]). In our hands, using an approx. 5-fold higher final dithiothreitol concentration than was used for the reduction of echicetin by Peng et al. [2], there was no remaining non-reduced echicetin and both subunits showed single peaks in the chromatogram (Figure 1). These results suggest that the functional activity of the reduced and alkylated echicetin β subunit reported by Peng et al. [2] and in an earlier report by the same group [1] was due to partial reduction of the protein. This suggests that the intersubunit disulphide bridge might be more susceptible to reduction than the other intrasubunit bonds. The susceptibility of the intrasubunit disulphide bonds might also be different in the α and β subunits of echicetin. This could also explain the difference in effects of alkylation on function between echicetin β [2] and jararaca GPIIb-binding protein β [25]. If the echicetin β was not fully reduced it could not be alkylated either. The reduced but not alkylated β -subunits might be able to refold rapidly once they are in a non-reducing environment, thus explaining the retention of biological activity.

Recently, some other snake C-type lectins that interact with platelet GPIIb were completely sequenced: *B. jararaca* GPIIb-binding protein [24] and alboaggregin B from *T. albolabris* [6]. The GPIIb-binding activity of the jararaca GPIIb-binding protein was also shown to be associated with the β subunit [24]. Unlike the jararaca GPIIb-binding protein and echicetin, which inhibit GPIIb-related platelet responses, alboaggregin B activates platelets, despite having a binding site in common, implying that the α subunit has a different role. In an attempt to learn more about the putative binding site for GPIIb on echicetin we have modelled the molecule on the basis of X-ray crystallographic data obtained with other C-type lectins (see Figure 3). It was already known that the C-type lectins of the botrocetin class do not require Ca^{2+} for their activity, nor do they necessarily show lectin activity by binding to sugar residues. It was therefore not

	1	50
MBP	SGKKFFVTN-HERMPFSK----	VKALCSEL---RGT
Echi α	DQDCLSGWSFYEGHCYQLFR-LK--TWDE----	AEKYC-NQWDGGHL
GPIb-BP α	DTPFECPSDWSHTRQYCYKFFQKE--SWDDRSEYDAERFCSEQAAGGHL	
Albo α	DCPSDWSFQYCYQI IKQLK--TWED----	AERFCMDQVKGHL
Echi β	NCLPDWSVYEGYCYKVFQKERM--NWAD----	AEKFCMKQVKGHL
GPIb-BP β	DCPSDWSFYGGHCYKLFQKRM--NWAD----	AENLCAQQRKESHL
Albo β	DCPSDWSYDLYCYRVFQEK--NWED----	AEKFTCQQTDSHL
Botro α	DCPSGWSSEYEGNCYKFFQKQ--NWAD----	AERFCSEQAAGGHL
Tf-IX/X-BP α	DCLSGWSSYEGHCYKAFQYK--TWED----	AERVCTEQAKGHL
Tf-IX-BP α	DCPSGWSSEYEGHCYKPFKLYK--TWDD----	AERFCTEQAKGHL
Ec-IX/X-BP α	DCLPGWSSHEGHCYKVFNEYK--TWKD----	AEKFCCKQKSGHL
Botro β	DCPPDWSSEYEGHCYRFFKEWM--HWDD----	AEEFCTEQQTGAHL
Tf-IX/X-BP β	DCPSDWSSEYEGHCYKPFSEPK--NWAD----	AENFTCQHQAGHL
Ec-IX/X-BP β	DCSSGWTAYGKHCYKVFDEPK--TWED----	AEKFCSEQAAGGHL
	51	100
MBP	VAIPRNAE-ENKAIQEV-----	AKTSAFLGITD--EVTEGQFMYVTG
Echi α	VSIESNAK-AEFVAQLISRLKPSAIEDRVWIGLRDRSKREQCQGHWTND	
GPIb-BP α	VSIES-DEEADFVAQLVAPNIGSKSY--VWIGLRLENKQCCSKWSY	
Albo α	VSIESY-REAVFVAQLSENKVTTKYD--VWIGLSVNVKQCCSSEWSG	
Echi β	VSFRN-SKEVDFMISLAFPLKME----LVWIGLS--DYWRDCYWEWSG	
GPIb-BP β	VSFHS-SEEVDFLLTFFILGPD----LYWTGLS--NIWNGCSFEWSG	
Albo β	VSFDS-SEEVDFVASKTFPVLKHD----LVWIGLG--SVWNACKLQWSG	
Botro α	VSIIYSKEKDFVGLVTNKIQSSDLY--AWIGLRVENKEKQCCSSEWSG	
Tf-IX/X-BP α	VSIES-SGEADFVAQLVTQNMRLDFY--IWIGLRVQKVKQCNSEWSG	
Tf-IX-BP α	VSIES-AGEADFVAQLVTENIQNTKSY--VWIGLRVQKKEKQCCSSEWSG	
Ec-IX/X-BP α	VSVES-SEEGDFVAKLISENLEKSHSIDFVWTLTYKGRWKQCCSSEWSG	
Botro β	VSFQS-KEEADFVRSLSLSEMLKGD----VWVIGLS--DVWNKCRFEWTDG	
Tf-IX/X-BP β	VSFQS-SEEADFVVKLAFQTFGHS----IFWVGLS--NVWNQCNQWNSNA	
Ec-IX/X-BP β	VSFRS-SKEADFVVTILTAQTESE----IVWVGLS--KIWNQCDWGWNTG	
	101	150
MBP	GRLTYSNWKDEPNHGSGE--DCVTIVDNG--LWNDISQASHTAVCEFFA	
Echi α	SFVHYEHVV-----PPT--KCFVLERQTEFRKWIADVNECFKFPFVCKAKIPR	
GPIb-BP α	SSVSyenLV-----RGNVKKCFALEKKQGFQKWNIDCEGNPFVCKFIRPR	
Albo α	SSVSyenLV-----KPLSKKCFVLKKGTEFRKWFNVACEQKHLFMCKFLRPR	
Echi β	AQLDYKAWD-----NERHCFAAKTTDN--QWMRKCSGEFYFVCKCPA	
GPIb-BP β	TKVYNNAW-----ASESECVASKTTDN--QWWSFCTRIQYFVCFEQA	
Albo β	TELKYNNAW-----SAESICITSKSTDN--EWLTRSCSRITYPFVCKFQA	
Botro α	SSVSyenV-----ERTVKKCFALEKDLGFVLWINLYCAQKNPFVCKSPPP	
Tf-IX/X-BP α	SSVSyenWI-----EAESKTCLEKETDFRQWNIYCGQNPVCEA	
Tf-IX-BP α	SSVSyenWI-----EAESKTCLEKETGFRQWNIYCGQNPVCEA	
Ec-IX/X-BP α	SKIKYQKWG-----KQPRKCLGLEKETFRQWNIYCEEPQRTCEI	
Botro β	MEFDYDDYY-----LIAEYECVASKPTNN--KWWIIPCTRFKNFVCFEQA	
Tf-IX/X-BP β	AMLRYKAW-----AEESYCVYFKSTNN--KWSRACRMMAQFVCFEQA	
Ec-IX/X-BP β	AALNYEAW-----AEAESYCVWFSTNN--EWSRCPCLFGHFVCKSPAW	

Figure 4 Sequence comparison of MBP and the α - and β -subunits of heterodimeric venom C-type lectins

MBP, rat MBP precursor [28] residues 124–238; Echi α , α subunit of *E. carinatus* echicetin (present study); Echi β , β subunit of *E. carinatus* echicetin [2]; GPIb-BP α and GPIb-BP β , α - and β -subunits of *B. jararaca* GPIb-binding protein [24]; Albo α and Albo β , α - and β -subunits of *T. albolabris* alboaggregin-B [6]; Botro α and Botro β , α - and β -subunits of *B. jararaca* two-chain botrocetin [4]; Ec-IX/X-BP α and Ec-IX/X-BP β , α - and β -subunits of *E. carinatus leucogaster* factor IX/X-binding protein [20]; Tf-IX/X-BP α and Tf-IX/X-BP β , α - and β -subunits of *T. flavoviridis* factor IX/X-binding protein [5,29]; Tf-IX-BP α , α -subunit of *T. flavoviridis* factor IX-binding protein [22]. Symbols: ^, residues in the 'hydrophobic surface' located where the Ca^{2+} -binding site is in the MBP structure; #, the MBP Ca^{2+} -binding domain truncated in the other structures; +, an additional hydrophobic residue present only in echicetin α -subunit and contributing to this surface. The GPIb-binding snake venom C-type lectins and those with other binding specificities are grouped separately in this sequence comparison.

surprising to see, from this model, that the Ca^{2+} -binding loop is truncated. A further interesting observation is that the residues in MBP that form the surface near this loop and are also involved in Ca^{2+} -binding are replaced by hydrophobic residues in the α subunits but not in the β subunits. On the basis of the conservation of most of the rest of the structure forming the typical C-type lectin fold, this truncated loop structure is a good candidate for at least part of the binding site for glycoprotein Ib. It has been shown that echicetin and jararaca GPIb-binding protein have inhibitory effects on platelet aggregation induced by vWf, whereas alboaggregin B induces platelet aggregation by binding to GPIb [1,6,25]. A comparison of the truncated loop sequence, which lies between an intrasubunit cystine bond and the intersubunit cystine bond and is therefore sterically very tightly constrained, shows some similarities between the GPIb-binding C-type lectins and distinct differences from the others such as botrocetin (see Figure 4). Thus the botrocetin β -chain contains the sequence DYDDYYL, similar to DLYDYYP within a thrombin/vWf-binding site in GPIb α [26], whereas the equivalent in echicetin β is DYKAWDN. Thus botrocetin might

partly simulate the structure of GPIb in forming a binding site for vWf. Similarly it might be expected that at least part of the sequence of echicetin binding to GPIb might resemble a sequence of vWf. Recently, a form of von Willebrand disease has been reported where vWf is present in a normal multimeric form but binding to platelet GPIb is absent [27]. The molecular defect was localized to a deletion of residues 629–639 (RMSRNFVRYVQ) of the A1 loop, which contains the GPIb-binding domain, suggesting that this sequence is critical for binding to GPIb. A search of the sequences of the known snake venom C-type lectins for similarities to this sequence indicated that the best match for the core seven-residue region was a sequence NSFVHYE, residues 88–94 within the truncated loop sequence of echicetin α . Another possible match lies within a more N-terminal loop of echicetin β , residues 45–51, SKEVDFM. There are no clear sequence similarities pointing to a related GPIb-binding sequence among the other GPIb-binding snake venom C-type lectins but it should not be forgotten that although all these proteins bind to the same domain of GPIb they might have optimized binding to different sequences. The fact that echicetin inhibits both

Figure 5 Computer model of the echicetin heterodimer

The position of the proposed GPIb-binding site is indicated. The position of the intersubunit disulphide bond is based on the high degree of conservation of this position within the botrocetin class of snake venom C-type lectins and not on direct determination. The position of the α -subunit in relation to the β -subunit apart from the cystine bridge is arbitrary and will change on binding.

thrombin- and vWf-induced platelet aggregation, whereas jararaca GPIb-binding protein inhibits only the latter, also supports this. The role of the α subunit in echicetin and jararaca GPIb-binding protein remains obscure although the heterodimer has an avidity some 100-fold higher than the β subunit alone. During the modelling it became clear that there were no obvious clues in the structure of each subunit to indicate the optimum interaction between the subunits. This has therefore been left flexible in the model (Figure 5) and might indeed provide part of the optimization of binding by adapting to the ligand in a sandwich mode. Clearly the linkage between the subunits has itself a major role in determining the orientation of the binding site. The presence of a hydrophobic sequence within the truncated loop in all the α subunits would argue against its being involved in the binding site, implying that in the α subunit the binding domain is elsewhere. The precise role of the α subunit thus remains unclear and will require X-ray crystallography of a complex with the ligand to provide an answer. Because echicetin and jararaca GPIb-binding protein, as far as is known, inhibit only GPIb function and only the β -subunit retained function after separation, it is possible that the α subunit binds to a domain within GPIb that, although enhancing the overall avidity, is not in itself critical for function. The fact that alboaggregin B activates platelets and is inhibited by echicetin [1] indicates that

it binds to a similar site but shows that alboaggregin B has other properties, such as being able to cross-link GPIb molecules in the same way as the natural ligand, vWf. Because the β -subunits are very similar this would implicate the α -subunit of alboaggregin B in the cross-linking.

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