

The disulfide bridge pattern of snake venom disintegrins, flavoridin and echistatin

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Received 9 July 1992

Flavoridin and echistatin, isolated from the venom of *Trimeresurus flavoviridis* and *Echis carinatus*, respectively, belong to the disintegrin family of integrin β_1 and β_2 inhibitors of low molecular weight RGD-containing, cysteine-rich peptides. Since disulfide bonds are critical for expression of biological activity, we sought to determine their location in these two proteins. In flavoridin, direct evidence for the existence of linkage between Cys⁸-Cys¹⁰ and between Cys¹¹ and Cys¹⁴ was obtained by analysis of proteolytic products, and indirect evidence suggests links between Cys⁸-Cys¹⁴ and Cys¹¹-Cys¹⁴. In echistatin, links between Cys⁸-Cys¹¹ and Cys¹⁰-Cys¹⁴ were identified by direct chemical analysis.

Inhibitor of integrin; Disintegrin; Disulfide bridge; Echistatin; Flavoridin

1. INTRODUCTION

Disintegrins represent a novel family of integrin β_1 and β_2 inhibitory proteins isolated from venoms of various vipers (family Viperidae) [1–4]. They interfere with biological processes such as cell adhesion to extracellular matrix, including adhesion of melanoma cells and fibroblasts to fibronectin [5,6], and are potent inhibitors of platelet aggregation (IC_{50} =20–200 nM) [1–11]. Analysis of the amino acid sequences of disintegrins [1–4, 6–12] shows that the tripeptide, RGD, which represents a cell attachment recognition sequence in a number of adhesive proteins [13], has been conserved in all but one of these molecules. The exception is barbourin, where lysine replacing arginine is thought to contribute to its integrin $\alpha II_b \beta_1$ antagonist specificity [4]. The appropriate spatial configuration of this recognition motif, determined largely by disulfide bridges, is essential for the inhibitory potency of disintegrins [3,5,7,14,15]. In a previous study, we established the disulfide bond pattern in albolabrin [15]. The purpose of this study was to determine the location of disulfide bridges in flavoridin, as well as in natural and synthetic echistatin, by isolation and sequencing of proteolytic fragments.

2. MATERIALS AND METHODS

Synthetic echistatin was purchased from Bachem Inc. (USA) and was used without further purification. Natural echistatin and flavoridin were purified by reverse-phase HPLC from the lyophilized

venom of *Echis carinatus* and *Trimeresurus flavoviridis* (Sigma Chemical Co., St. Louis, MO), respectively, using a Vydac C18 column (Hesperia, CA) (0.44 x 25 cm, 300 Å pore size) and eluting with a mixture of 0.1% (v/v) TFA in water (solution A) and acetonitrile (solution B). The column was first eluted isocratically (100% A) for 10 min, followed by a linear gradient up to 80% B in 50 min. Active peptides were identified by their ability to inhibit ADP-induced platelet aggregation as described [15].

Echistatin, either natural or synthetic, and flavoridin, (2 mg/ml in 100 mM NH_4HCO_3 , pH 8.0) were digested with TPCK-trypsin (Sigma) at an enzyme:substrate ratio of 1:50 (w/w) for 4 h at 37°C. Digestion of echistatin was stopped with formic acid (final concentration 30% (v/v)), and the tryptic peptides were separated by reverse-phase HPLC on a Lichrospher 100 RP-18 (5 μ M particle size) (Merck, Darmstadt) column equilibrated in a mixture of 0.1% (v/v) TFA in water (solution A) and in acetonitrile (solution B) (95% A/5% B) and eluted at 1 ml/min, at first isocratically for 5 min and then followed by a linear gradient up to 40% B in 70 min.

The enzyme in the tryptic digest of flavoridin was heat-inactivated (100°C for 5 min), endoproteinase Asp-N (Boehringer-Mannheim, Indianapolis, IN) was added up to a final enzyme:substrate ratio of 1:25 (w/w) and incubated 6 h at 37°C. Thereafter, the resulting peptides were separated as above. Elution conditions were: 5 min isocratically (100% A) followed by a linear gradient up to 30% B in 90 min.

The isolated peptides were characterized by amino acid analysis (after acid hydrolysis with 6 N HCl for 24 h at 110°C using a Biotronic (amino acids analyzer) and amino-terminal sequence analysis (using an Applied Biosystems, San Francisco, CA) gas-phase sequencer model 473A. Mass spectra were recorded with a mass spectrometer MAT 900 (Finnigan MAT, Bremen, FRG) equipped with liquid secondary ion ionization system.

3. RESULTS

3.1. Disulfide bridge pattern of flavoridin

Proteolytic digestion of flavoridin with trypsin and endoproteinase Asp-N gave nine fractions (Table I).

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Table I

Amino acid sequence of flavoridin [1] and of peptides obtained by reverse-phase HPLC after its digestion with trypsin and endoprotease Asp-N

Flavoridin:									
GEECDGSPSNPCCDAATCKLRPGAQCAAGLCCDGGCRFKKKRTICRIARGSPFPDRCTGLSHDCPRHMDL	1	10	20	30	40	50	60	70	
									Trypsin, Asp-N
F-1:	30	FK			39				
	47	IAR			48				
	70	DL			70				
F-2:	1	GEEC			3				
	15	DAATCK			20				
F-3:	38	FKKK			41				
	68	WH			69				
F-4:	21	LRPGAQ			25				
F-5:	27	CA			28				
	32	CC			33				
	58	CTGLSH			63				
F-6:	43	TICR			46				
	64	DCPR			67				
F-7:	58	TGLSH			63				
F-8:	8	DGSPSNPCC			14				
	34	DGCR			37				
F-9:	21	LRPGAQCAAGLCC			33				
	58	C							

Table II

Amino acid sequence of echistatin and of peptides obtained by its digestion with TPCK-trypsin (the order of some products has been altered to show a degradation pathway from T-3 through T-5)

Echistatin:									
ECESGPCCRNCKFLKEGTICKRARGDDDDYCNKGTCDPRHNPCKGPAT	1	10	20	30	40				
									TPCK-Trypsin
T-1:	42	NPHK			45				
	46	GPAT			49				
T-2:	13	FLK			15				
T-3:	1	ECESGPCCRNCKFLKEGTICK			21				
	25	GDDDDYCNKGTCDPR			41				
T-6:	1	ECESGPCCRNCK			12				
	16	EGTICK			21				
	25	GDDDDYCNKGTCDPR			41				
T-4:	1	ECESGPCCRNCK			9				
	10	NCK			12				
	25	GDDDDYCNKGTCDPR			41				
T-5:	1	ECESGPCCRNCK			9				
	10	NCK			12				
	16	EGTICK			21				
	25	GDDDDYCNKGTCDPR			35				
	36	TEDCPR			41				

Two fractions, F-2 and F-6 ($M + H^+ = 979.1$), each contained two sequences in a 1:1 molar ratio, indicating the existence of disulfide bonds between Cys⁴ and Cys¹⁹ in fraction F-2, and between Cys⁴³ and Cys⁶³ in fraction F-6. On the other hand, fractions F-5, F-8 and F-9 each contained four cysteine residues involved in the formation of two disulfide bridges. Although at this stage the actual pairing could not be established, the fact that a disulfide bond between adjacent cysteines is sterically hindered limits to four the actual pairing possibilities. Thus, Cys⁶ cannot be paired with Cys³⁶, since such a pairing would leave two adjacent cysteines, Cys¹³ and Cys¹⁴, unpaired. The pairing of Cys²⁷-Cys³⁸ is also forbidden, since this would leave Cys³² and Cys³³ unpaired. Among the remaining possibilities, bonding between Cys⁶-Cys¹⁴, Cys¹³-Cys³⁶, Cys³²-Cys³⁸, and Cys²⁷-Cys³³ would be consistent with the recently reported NMR solution structure of kistrin [16], a similar disintegrin [3].

3.2. Disulfide bridge pattern of echistatin

The tryptic peptides of synthetic echistatin are shown in Table II. T-1, T-2 and T-5 were final digestion products, while T-3 was a partial digestion product that gave rise to T-6, T-4 and T-5 in that order. This established that the first cleavage sites occur at bonds K⁴¹-R⁴², R²²-A²³, R²⁴-G²⁵, R⁴¹-N⁴² and K⁴⁵-G⁴⁶, indicating that the RGD recognition motif and the C-terminal tail are

both readily exposed; the other bonds, K¹²-F¹³, K¹⁵-E¹⁶, R⁹-N¹⁰ and K³⁴-T³⁵, were presumably less accessible. The final tryptic degradation product, T-5 (Table II) was composed of five fragments cross-linked by four disulfide bonds giving rise to several possibilities for disulfide bridges.

To establish the actual disulfide bridge arrangement in echistatin, T-5 was further degraded with elastase, and the resulting peptides were isolated by reverse-phase HPLC. This yielded a set of successively degraded products from which a degradation pattern as well as the position of disulfide bridges could be deduced (Table III). On degradation of T-5/E-7 to T-5/E-6, Cys²⁰ and Cys³⁹ both disappeared indicating that Cys²⁰ and Cys³⁹ were disulfide linked. This confirms NMR studies [17-21]. Cleavage of T-5/E-5 ($M + H^+ = 2,239.8$) to T-5/E-4 resulted in the loss of Cys⁸ and Cys³⁷ indicating that they were disulfide bonded. Analysis of fragment T-5/E-1 ($M + H^+ = 1,311.1$) did not show whether Cys⁷ was paired to Cys¹¹ or Cys³². The second possibility would agree with recently reported ¹H NMR studies of synthetic echistatin in solution [16-20]. Furthermore, an S-S bond between Cys² and Cys¹¹ has been described [18]. The resonance studies could not distinguish between the pairing possibilities Cys⁸-Cys³⁷; Cys⁷-Cys³², and Cys⁸-Cys³²; Cys⁷-Cys³⁷. Our protein-chemical data established the Cys⁸-Cys³⁷ linkage, which was previously predicted on the basis of the disulfide bridge pattern reported for albolabrin [14].

Table III

Amino acid sequence of the digestion products of peptide T-5 by porcine pancreatic elastase (a degradation pathway for T-5/E-8 through T-5/E-1 can be followed)

T-5/E-1:	7	CCR	8
		C	20
		C	11 or 32
	38	TCDCPR	41
T-5/E-2:	18	EGT1	19
T-5/E-3:	25	QDDHDDY	31
T-5/E-4:	1	ECESGPC	6
	10	NCK	12
	25	QDDHDDYC	32
T-5/E-5:	1	ECESGPCC	8
	10	NCK	12
	25	QDDHDDYC	32
	37	C	
T-5/E-6:	1	ECESGPCCR	9
	10	NCK	12
	25	QDDHDDYCNGK	35
	38	TCQ	38
T-5/E-7:	1	ECESGPCCR	9
	10	NCK	12
	20	CK	21
	25	QDDHDDYCNGK	35
	38	TCQC	39
T-5/E-8:	1	ECESGPCCRC	9
	10	NCK	12
	16	EGTICK	21
	25	QDDHDDYCNGK	35
	38	TCQC	39

When natural echistatin was proteolytically degraded in the same way as the synthetic molecule, a very minor component (estimated to represent less than 5%), which

contained the peptides 'ECESG' and 'GDD-MDDYC'³² disulfide bridged ($M + H^+ = 1,454.5$) was found. Whether this peptide resulted from limited disulfide bridge rearrangement during isolation and/or degradation of the protein, or whether it reflects the existence of a small echistatin population with an alternative disulfide bridge pattern (which interestingly would be similar to the one found in albolabrin [15]) cannot be concluded from our data. Nevertheless, computer modelling of this alternative pairing showed that it would not introduce significant changes into the conformation of the RGD loop.

4. DISCUSSION

Since disulfide bridges are essential for expression of the biological activity of disintegrins [3,4,11,13,14], we were interested in the disulfide bridge patterns in these peptides. Isolation, amino acid sequencing and analysis of proteolytic fragments of flavridin (Table I) and echistatin (Tables II and III) enabled us to identify some disulfide bridges (Fig. 1). The disulfide bridge pattern of albolabrin as previously described [15] was added for comparison. We identified two S-S bridges in flavridin, and the other ones were deduced. Our observations on echistatin confirm the location of two disulfide bridges (Cys²-Cys¹¹ and Cys²⁰-Cys³⁹) previously proposed on the basis of NMR studies [17,18]. We were able to add the location of one more (Cys⁸-Cys¹⁷) based on our protein chemistry data. Bond Cys⁷-Cys³² was deduced since these were the only remaining cysteines.

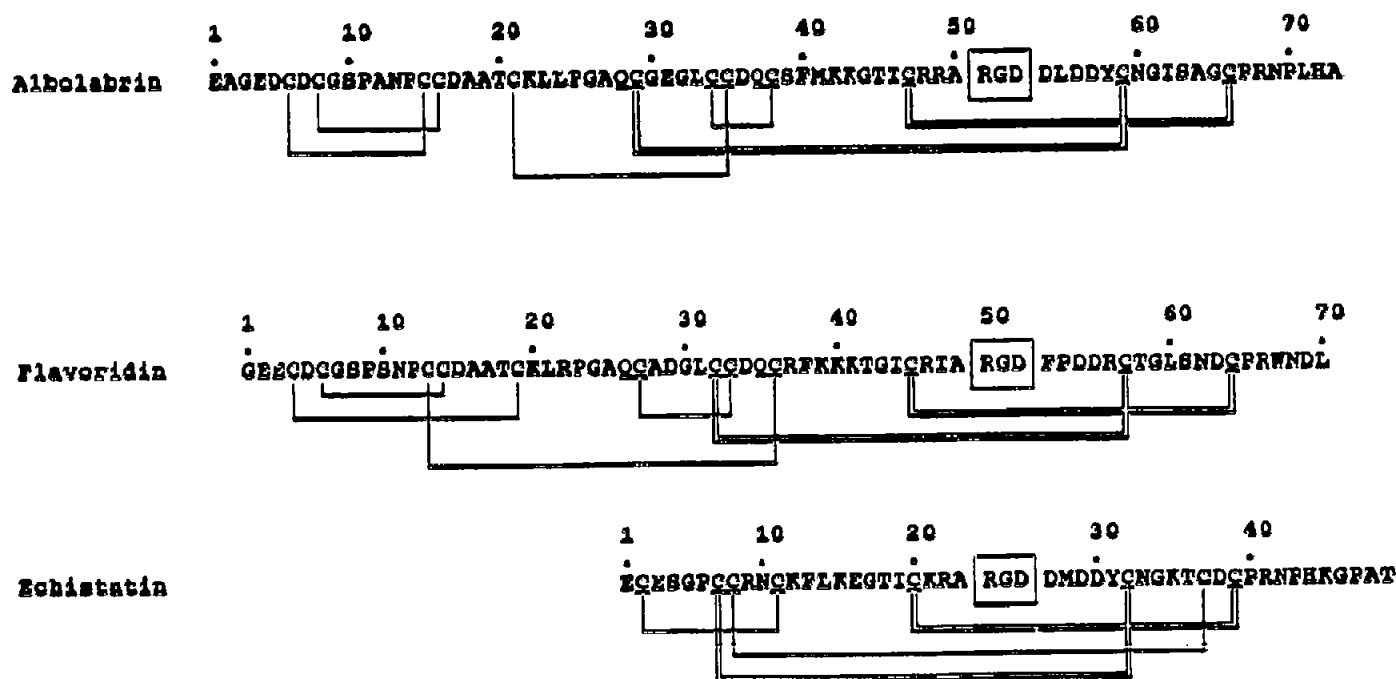


Fig. 1. Schematic representation of the structure of three disintegrins. Disulfide bonds that are conserved or semi-conserved are shown by double lines. The other bonds are shown by single lines.

In flavoridin and echistatin, as well as in albolabrin [15], the disulfide bonds form a complex pattern of overlapping loops and loops within loops (Tables I-III, Fig. 1). This pattern extends from the first to the last cysteine in the peptide, and leaves only a few N-terminal (one to five) and C-terminal (six to ten) amino acids beyond these loops [1-5, 6-10]. In all three disintegrins, two disulfide bridges form an interesting motif with respect to the RGD sequence. A domain containing the RGD sequence is defined by a combination of two disulfide bonds. One is formed between the cysteine closest to the RGD sequence on the N-terminal end of the peptide and the last cysteine in the peptide. This bond is highly conserved. The other is between the cysteine closest to the RGD sequence on the C-terminal end of the molecule and one cysteine of the N-terminal segment.

The formation of these two disulfide bridges has two consequences, (i) delineation of an RGD-containing domain that has 11 amino acids between the cysteines, and (ii) formation of a shorter (6 amino acid) segment of peptide that limits the distance between the S-S bridges defining the RGD-containing domain (a 'retainer' segment). Obviously this arrangement throws the RGD-containing domain into a loop, a finding consistent with observations made by NMR [17-21]. This motif depends on the presence of two or more cysteines, both C-terminal and N-terminal to the RGD sequence, and the formation of at least two trans-RGD disulfide bonds. All disintegrins sequenced to date have two cysteines C-terminal to the RGD sequence, except for echistatin and cristostatin which have three C-terminal cysteines. All sequenced disintegrins have more than two cysteines N-terminal to the RGD sequence, with the number depending on the length of the peptide. If the 'retainer' segment is able to change its length by forming a secondary structure, this arrangement might provide a means for varying the shape of the loop, thereby influencing the configuration of the RGD sequence and possibly the biological activity of the peptide.

Elucidation of the disulfide bridge patterns of flavoridin and echistatin (Fig. 1) provide information for revising our previous prediction that the disulfide bond pattern would be the same in all disintegrins [15]. It is noteworthy that flavoridin and kistrin [16] have identical patterns of S-S bridges [16]. The differences between albolabrin compared to flavoridin and kistrin probably depend on differences in folding which would bring different cysteines within bonding distance of each other. Differences in folding patterns probably depend on differences in amino acid sequences in the peptides.

Three peptides with similar ability to antagonize the platelet fibrinogen receptor have been isolated from other sources and sequenced. They have little resemblance to the disintegrins in either amino acid composition or arrangement of cysteines. Two peptides, decorsin and ornatin, isolated from leeches, have only one cysteine C-terminal to the RGD sequence [22,23]. Thus,

only one trans-RGD disulfide bond can be formed, i.e. an RGD-containing loop in these peptides must be defined by one rather than two disulfide bonds. The other peptide, mambin, was isolated from the venom of a snake belonging to the family *Elapidae*. Mambin differs from leech peptides as well as disintegrins [24]. While mambin has three cysteines C-terminal and five N-terminal to the RGD sequence, McDowell et al. [24] suggested that the RGD-containing loop is defined by one disulfide bond, that between Cys⁴³-Cys⁴⁴. The influence of these different patterns of disulfide bonds on the conformation of the RGD-containing loop and on biological functions of the various peptides will help to establish structure-function relationships.

Acknowledgements: The provision of a Max-Planck-Gesellschaft fellowship to J.J.C. is gratefully acknowledged. This study was also supported by a National Institutes of Health Grant HL 45486 (S.N. and G.J.S.).

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