Echistatin disulfide bridges: Selective reduction and linkage assignment

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Abstract

Echistatin is the smallest member of the disintegrin family of snake venom proteins, containing four disulfides in a peptide chain of 49 residues. Partial assignment of disulfides has been made previously by NMR and chemical approaches. A full assignment was made by a newly developed chemical approach, using partial reduction with tris-(2-carboxyethyl)-phosphine at acid pH. Reduction proceeded in a stepwise manner at pH 3, and the intermediates were isolated by high performance liquid chromatography. Alkylation of free thiols, followed by sequencer analysis, enabled all four bridges to be identified: (1) at 20 °C a single bridge linking Cys 2–Cys 11 was broken, giving a relatively stable intermediate; (2) with further treatment at 41 °C the bridges Cys 7–Cys 32 and Cys 8–Cys 37 became accessible to the reagent and were reduced at approx. equal rates; (3) the two bicyclic peptides produced in this manner were less stable and could be reduced at 20 °C to a peptide that retains a single bridge linking Cys 20–Cys 39; and (4) the monocyclic peptide can be reduced to the linear molecule at 20 °C. Some disulfide exchange occurred during alkylation of the bicyclic intermediates, but results unambiguously show the pattern to be [2-11; 7-32; 8-37; 20-39]. A comparison is made with kistrin, a longer disintegrin whose disulfide structure has been proposed from NMR analysis.

Keywords: analysis; disulfide; echistatin; reduction; structure; TCEP

Disintegrins are small proteins isolated from snake venoms (Gould et al., 1990). Their biological activity is based on the Arg-Gly-Asp (RGD) sequence by which they inhibit various reactions related to cell adhesion (Pierschbacher & Ruoslahti, 1984), including the aggregation of platelets involved in blood coagulation. Echistatin from the venom of *Echis carinatus* (Gan et al., 1988) is the smallest (49 residues) member of the family and has been the focus of intense activity. Several laboratories have used NMR to investigate the three-dimensional conformation of the protein and agree that the RGD sequence is located at the tip of an extended hairpin loop (Chen

et al., 1991; Dalvit et al., 1991; Saudek et al., 1991a,b; Cooke et al., 1991, 1992).

Two of the four disulfides, linking Cys 2–Cys 11 and Cys 20–Cys 39 were initially assigned from the NMR data (Chen et al., 1991; Saudek et al., 1991b). The other two bridges involve connections among Cys residues 7, 8, 32, and 37, with one pattern [7-8; 32-37] being definitely excluded. Distinguishing between arrangements [7-32; 8-37] and [7-37; 8-32] poses a familiar problem for chemical approaches, because adjacent Cys residues are not readily cleaved to give distinctive peptides. Further refinement of the NMR data favors the [7-32; 8-37] arrangement, but the authors refer to their conclusions as predictive rather than definitive (Cooke et al., 1992).

In a recently developed approach to solving disulfide structures of tightly clustered cystines (Gray, 1993), partial reduction by TCEP (Burns et al., 1991) is used at acid pH to generate a series of intermediates containing both disulfides and thiols. Separation of these intermediates by HPLC is followed by alkylation, and the labeled thiols are identified by sequencer analysis. This method was applied to echistatin, and all four disulfides were unambiguously assigned as [2-11; 7-32; 8-37; 20-39]. Reduction at 20 °C

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Abbreviations: BPTI, bovine pancreatic trypsin inhibitor; HPLC, high performance liquid chromatography; TCEP, tris-(2-carboxyethyl)-phosphine; TFA, trifluoroacetic acid; 4-VP, 4-vinylpyridine. Amino acids are indicated by the standard three- or one-letter abbreviations; Cys(Cam), S-carboxamidomethylcysteine; Cys(Pe), S-pyridylethylcysteine. **Oxidation status of Cys residues:** Square brackets enclose pairs of numbers separated by semicolons; numbers within a pair are separated by a hyphen when the residues are connected by a disulfide bridge, or by a comma when the residues are present as thiols. Thus [2, 11; 7-32] indicates a peptide with thiols on residues Cys 2 and Cys 11, and a disulfide linking residues Cys 7 and Cys 32.

is highly selective for disulfide [2-11], the singly reduced peptide being resistant to further cleavage by TCEP until 40 °C. At this temperature disulfides [7-32] and [8-37] become accessible to reduction. Once either of these is broken, the other is readily reduced at room temperature, along with the fourth bridge [20-39].

As pointed out by Cooke et al. (1992), this pattern of bridging is not fully concordant with structures proposed for kistrin (Adler et al., 1991, 1993), albolabrin (Calvete et al., 1991), and flavoridin (Calvete et al., 1992), all of which are longer disintegrins with six bridges. Given the difficulty of disulfide assignment in these tightly bridged molecules, great caution must be exercised in making comparisons among structures that are not yet rigorously defined.

Results

Partial reduction of echistatin

Trial experiments with samples of 200 pmol of peptide showed that echistatin reduced slowly at room temperature to give a single major product (EchiR1). A larger scale reduction of 15 nmol is shown in Figure 1a, where 43% of the starting material had been converted to EchiR1 during a 20-min incubation with 10 mM TCEP. This product was identified as a singly reduced species because of its clean reoxidation to native echistatin by mild

iodine treatment (0.5 mM I₂, pH 2, 20 s, room temperature). Alkylation of EchiR1 by direct addition of 4-VP gave a single peak with a slight shoulder. Sequencer analysis of this material for 21 cycles showed clean labeling of Cys 2 and Cys 11 (Fig. 2a). Although the run examined only five of the eight Cys residues, the results point strongly to EchiR1 being the molecule in which a single bridge linking Cys 2–Cys 11 had been opened. This interpretation is fully consistent with all further experiments.

Further reduction of EchiR1

More vigorous treatment with TCEP gave a series of peaks eluting close to the fully reduced peptide (EchiR4). Figure 1b shows the profile from a reduction of EchiR1 carried out at 41 °C for 20 min. Three major intermediates between EchiR1 and EchiR4 were assigned as EchiA–EchiC; other minor peaks and shoulders were not investigated further. Adequate amounts of these intermediates were obtained by a simple recycling process: EchiR1 was treated again with TCEP during HPLC equilibration, while the other peaks were collected and stored on dry ice. In this way a complete cycle could be carried out in less than 1 h, and six cycles were performed in succession. Reduction time was limited to 2–5 min, so that only about 15% of EchiR1 was converted to a mixture of EchiA–C, and little was lost by conversion to EchiR4. With these short reaction times, it was clear that EchiA and EchiB are immediate products of EchiR1 reduction (and therefore have two bridges open), whereas EchiC results from the further reduction of EchiA and EchiB (and therefore has three bridges open). Direct conversion of both EchiA and EchiB to EchiC was observed on further brief treatment with TCEP.

Alkylation of EchiB

Pooled fractions of EchiB were diluted with 0.1% TFA and repurified as a single batch on HPLC. Alkylation by iodoacetamide, using the rapid procedure, was straightforward. A single major product (55%) was obtained, plus minor satellites (Fig. 1c). Sequencer analysis (41 cycles) of peptide from the main peak is shown in Figure 2b. In addition to the expected label at positions 2 and 11, there is a significant increase in Cys(Cam) at cycles 7 and 32. Yields of Cys(Cam) at these positions were similar to yields of other amino acids at the corresponding stage of the sequencer run. At cycles 20, 37, and 39 there was no increase in Cys(Cam). This rules out significant labeling of Cys 20 and Cys 37, but the sequencing yield was too low to make a strong statement about Cys 39. Marked carryover developed early in the sequencer run, as is shown by tailing of the signal from Cys 11. Allowing for this in the data for cycles 7–10, one can estimate that Cys 8 was labeled to about 20% of the extent that Cys 7 was labeled. Taking the earlier result into account, the most econom-

Fig. 1. HPLC purification of products from partial reduction and alkylation of echistatin. Buffer A = 0.1% TFA in H₂O; B = 0.092% TFA in acetonitrile/water (60/40 v/v). Gradients: a and b, 15–45% B/30 min; c and d, 20–40% B/20 min. Peptides eluting at the indicated positions are referred to as EchiN, EchiR1, etc. **a:** Native echistatin, N, was treated with 10 mM TCEP at pH 3, 20 min, 22 °C. **b:** EchiR1 was treated with 10 mM TCEP at pH 3, 20 min, 41 °C. **c:** Alkylation of EchiB with iodoacetamide. **d:** Alkylation of EchiA with iodoacetamide. Material from peaks EchiB* and EchiA* was used for sequencer analyses (see Fig. 2).

Fig. 2. Protein sequencer analysis of partially reduced and alkylated echistatin derivatives. Ordinate represents yield (pmol) of the appropriate Cys derivative at each stage of analysis. Values for Cys(Cam) are corrected for approx. 25% partial deamidation to Cys(Cm), but otherwise all values are uncorrected. **a:** EchiR1 (Fig. 1a) was alkylated with iodoacetamide and analyzed for 21 cycles. **b:** EchiB (Fig. 1b) was alkylated with iodoacetamide, and EchiB* (Fig. 1c) was analyzed for 41 cycles. **c, d:** EchiA (Fig. 1b) was alkylated with iodoacetamide, the product EchiA* (Fig. 1d) was reduced and alkylated with 4-VP, and the resulting peptide was analyzed for 42 cycles.

ical interpretation of the data is that EchiB is reduced at disulfides Cys 2–Cys 11 and Cys 7–Cys 32 and that labeling of Cys 8 occurred following thiol–disulfide exchange during alkylation.

Double alkylation of EchiA

This peptide was more labile than EchiB. When unmodified EchiA was allowed to stand at room temperature,

disulfide exchange occurred within a few hours, even at pH 2: several new peaks were generated, which eluted later than EchiA on HPLC. Freshly prepared peptide labeled with iodoacetamide gave the profile shown in Figure 1d. The main peak accounted for only 45% of the absorbance, while other significant peaks eluted later. Samples alkylated after partial exchange had occurred showed a major decrease of the principal peak and a corresponding increase of the later peaks. It was thus con-

cluded that the main peak represented correctly labeled (unrearranged) peptide. Freshly prepared EchiA was therefore alkylated, and the main peak of EchiA(Cam)₄ was purified by HPLC. To improve sequencer analysis, the peptide was fully reduced by incubation with TCEP at pH 8, and the newly exposed thiols were alkylated with 4-VP. An extended cleavage step at Pro 6 also led to a better repetitive yield and less carryover. Sequencer analysis (Fig. 2c,d) unambiguously located Cys(Cam) at cycles 2, 8, 11, and 37, while Cys(Pe) was found at cycles 7, 20, 32, and 39. The peptide showed minimal cross-labeling of these positions.

Discussion

Echistatin is the shortest member of the family of RGD-active proteins that inhibit cell-cell adhesion. Its amino acid sequence (Fig. 4) was reported by Gan et al. (1988), its synthesis by Garsky et al. (1989), and its three-dimensional structure by Saudek et al. (1991b). Although the latter work defined the overall chain fold, the four disulfides could not be fully assigned. Two bridges were positively located between Cys 2-Cys 11 and Cys 20-Cys 39. The other two involved an adjacent pair, Cys 7 and 8, plus Cys 32 and Cys 37. Either of two patterns ([7-32; 8-37] or [7-37; 8-32]) fitted equally well with the NMR data. Cooke et al. (1992) refined their earlier NMR data (Cooke et al., 1991) and presented evidence favoring the [7-32; 8-37] arrangement. The work reported here fully supports this prediction.

Bridging pattern of echistatin

Taken together, the partial reduction results described above provide a clear case for the disulfide structure of echistatin being [2-11; 7-32; 8-37; 20-39]. Although some disulfide exchange occurred during alkylation, it did not preclude making an unambiguous choice of which labeled peptides were the important ones; analyzing a rearranged peptide would almost certainly have led to inconsistencies in the results. Redundancy in the sequencer data reinforces the basic conclusions: (1) Labeling of EchiA and

EchiB is actually sufficient to define the whole pattern. Cys 2 and Cys 11 are labeled in both peptides and therefore represent the bridge opened in their common precursor EchiR1; bridges [7-32] and [8-37] are defined by the unique labeling in each of the two peptides; Cys 20 and Cys 39 were unlabeled, indicating that bridge [20-39] remained closed in both. (2) Labeling of Cys 2 and Cys 11 in EchiR1 strongly supports the conclusion, even though this particular experiment did not examine all eight Cys residues. (3) Clean reoxidation of EchiR1 to native peptide confirms that it is a singly reduced peptide, and that it has not rearranged. (4) Precursor-product relationships were established for the various intermediates, as in Figure 3.

Note that it is not always possible to deduce four bridges from two labeled peptides as in (1). It can only be done when the peptides are two-bridged intermediates having one bridge the same. Because the simple "chase" experiments had identified EchiA and EchiB as being properly related in this way, their suitability was not in doubt. Injudicious choice of intermediate peptides for study, or force of circumstance, could have resulted in three or even four peptides being needed to define the whole pattern. In carrying out analyses such as this, one should also consider using two different secondary labels. If EchiB had been secondarily labeled with iodoacetic acid, as EchiA had been labeled with 4-VP, a *single sequencer run on a mixture* of the two peptides would have sufficed. Not only would this have saved a long (42-cycle) sequencer run, but the actual analysis could well have been improved by having a higher total loading of peptide.

Under the conditions used there was a definite pattern to the reduction process, shown schematically in Figure 3. Opening of the Cys 2-Cys 11 bond was highly selective at room temperature, giving a stable product, EchiR1. This resisted further reduction and could be alkylated cleanly even under very nonforcing conditions. As the mixed thiol-disulfide form, it has been stored at -76 °C for almost a year with <5% exchange or reoxidation. This behavior is reminiscent of disulfide [14-38] of BPTI (Kress & Laskowski, 1967) and disulfide [181-189] of somatotropin (Buckwalter et al., 1992), which can be reduced selectively.

Fig. 3. Preferred pathway for reduction of echistatin by TCEP (10 mM, pH 3). Peptides are identified according to Figure 1a,b. Reduction is shown proceeding from fully oxidized native echistatin (N) to fully reduced (R4). Bold numbers by the arrows indicate which disulfide is reduced at that step. Note that the second step (reduction of EchiR1 to either EchiA or EchiB) occurs readily only at higher temperatures, whereas all others take place at 20 °C.

Further reduction of EchiR1 did not occur readily until the temperature was raised to 41 °C, when the central pair of disulfides became accessible. Both of these reacted with TCEP at comparable rates to give EchiA [2, 11; 7-32; 8, 37; 20-39] and EchiB [2, 11; 7, 32; 8-37; 20-39]. Not surprisingly, the close spatial juxtaposition of Cys 7, 8, 32, and 37 led to rapid thiolate-disulfide exchange reactions as the pH was raised during alkylation, especially with EchiA. One attempt was made to lessen the disulfide exchange but did not prove helpful: rather than reducing EchiR1 itself, the free thiols on Cys 2 and Cys 11 were first alkylated with iodoacetamide to give EchiR1(Cam)₂. This molecule proved to be more resistant and had to be heated above 50 °C to get further reduction.

Both EchiA and EchiB could be reduced readily to EchiR4 at room temperature, apparently through the same intermediate EchiC. This latter peptide was not investigated further, but its arrangement should be [2, 11; 7, 32; 8, 37; 20-39]. It is likely that this intermediate, lacking any special proximity of thiols and disulfide, would alkylate cleanly with little exchange.

Structure and stability of echistatin

Saudek et al. (1991b) describe the molecule as "... a bundle of loops containing no regular secondary structure. The disulfide bridges stabilize the central rigid part from which an irregular β -hairpin and the C-terminus protrude." The active site RGD is at the tip of the hairpin loop.

My results suggest that disulfide [2-11] is not critical for stabilizing the molecule. This bridge is located at the far end of the peptide from the active site and can be reduced selectively. Echistatin treated in this way is relatively stable to further reduction, and the next two bridges are not opened until the peptide is partially unfolded by heating to 41 °C, or even higher (>50 °C) if the thiols on Cys 2

and Cys 11 are alkylated with iodoacetamide. The bridges that open at this second stage are the close pair [7-32] and [8-37]. Once either of these is reduced, the other is readily attacked and reduction goes rapidly to completion. It is noteworthy that Saudek et al. (1991a) found an irreversible change in the CD spectrum of echistatin at about the same temperature at which the second and third disulfides of EchiR1 become accessible to reduction. The last bridge to be reduced (Fig. 3) is that linking Cys 20-Cys 39, one arm of which is involved in a very stable H-bond at the border between the central core and the mobile part of the hairpin. This bridge is also the only one that was assigned equivalently in the analyses of albolabrin and kistrin described below.

Comparison with NMR results and other disintegrins

The disintegrins are of great interest because of their implications for therapy of coagulation disorders. Structural analysis by NMR has been carried out for echistatin by four independent groups (Chen et al., 1991; Dalvit et al., 1991; Saudek et al., 1991a,b; Cooke et al., 1991, 1992) and for one larger protein, kistrin (Adler et al., 1991, 1993). With echistatin, two of the four disulfides (2-11 and 20-39) could be assigned directly by this approach (Chen et al., 1991; Saudek et al., 1991b). Beyond this, refinement of the NMR data with various sets of disulfide constraints lends statistical support in favor of [2-11; 7-32; 8-37; 20-39] (Cooke et al., 1992; Pitzemberger, pers. comm.). This is the pattern determined in the present work, and is illustrated in Figure 4.

Seven of the eight half-cystine residues of echistatin have exact counterparts in the long disintegrins, with only Cys 37 being unmatched. Conservatively, then, one expects its partner Cys 8 to be involved in linkage to the amino-terminal extension in the long disintegrins, leav-

Kistrin

Echistatin

Fig. 4. Disulfide bridge patterns proposed for echistatin (this work; also Cooke et al., 1992) and kistrin (Adler et al., 1991, 1993). Linkages above the line of text indicate the two bridges assigned equivalently in both proteins, whereas those below the line indicate nonequivalent bridges.

ing the other three bridges unaltered. Available evidence from NMR analysis of kistrin (Adler et al., 1991, 1993), however, gives a disulfide assignment that is not compatible with this arrangement (Fig. 4). Bridges equivalent to 7-32 and 20-39 are conserved, but the homologue of Cys 2 is connected to the Cys 8 equivalent, and the Cys 11 homologue is linked to the amino-terminal extension. Does this truly reflect a "disulfide bond rearrangement," in the evolutionary sense proposed by Cooke et al. (1992)? I believe it is premature to draw this conclusion, given the extraordinary difficulty in making rigorous assignments with these tightly bridged proteins. Adler et al. (1993) made a persuasive case for their assignments, but noted that there was at least one peptide isolated that did not fit the pattern.

Chemical evidence on the long disintegrins is also difficult to evaluate. Calvete and coworkers have made partial assignments for two proteins, albolabrin (Calvete et al., 1991) and flavoridin (Calvete et al., 1992; also echistatin). Very prolonged enzymatic digestions were made to obtain fragments suitable for analysis by sequencing or mass spectrometry, with no consideration given to the possibility of "disulfide bond rearrangement" in the chemical sense. Although peptides supporting the partial assignments were isolated, a reader cannot judge whether they truly represent the native molecule or some rearranged product. With such intrinsically stable proteins, it is expected that non-native forms arising by disulfide exchange would be less stable and thus overrepresented in the digestion fragments. The question is acute in the present case, because the patterns deduced in this way for albolabrin and flavoridin differ from each other despite the exact correspondence of all 12 half-cystine residues. Although the partial pattern derived for flavoridin (Calvete et al., 1992) agrees with that proposed by Adler et al. (1991, 1993) for kistrin, and the pattern for echistatin agrees with the one assigned here and by Cooke et al. (1992), in the absence of critical data there is no way of assessing the correctness of the assignments. Disulfide exchange is a real problem. Even in the presence of disulfide exchange inhibitors such as *N*-ethylmaleimide, trypsin digestion of native echistatin gave rise to peptide fragments that could not be interpreted in terms of a unique linkage pattern (Garsky, pers. comm.). In related work with a small peptide toxin from the snail *Conus magus*, there is clear evidence of disulfide rearrangement during trypsin digestion (Craig et al., 1993).

Thus, I believe an open mind should be kept on the bridging pattern of long disintegrins until the possibility of disulfide exchange has been addressed. In all work on this and similar molecules, it would clearly be advantageous to have rigorously known disulfides as part of the input constraints when calculating structures from NMR data. This could be done using the partial reduction strategy described here, but some modifications to the method would be necessary. Echistatin, with 49 amino acids, is

close to the limit for reliable analysis by direct sequencing. Longer proteins such as kistrin will need to be fragmented and analyzed by some combination of sequencing and/or mass spectrometry. To minimize the risk of disulfide exchange, fragmentation would seem best to be done after the partial reduction and labeling, because the exchange that does occur during these steps is more easily recognized as such. The larger proteins may also show increased resistance to attack by TCEP.

Materials and methods

Echistatin

Echistatin was a gift of Dr. M. Verlander, Bachem, Inc., Torrance, California.

Chemicals

TCEP was synthesized by the method of Burns et al. (1991). 4-VP was purchased from Aldrich, distilled under vacuum, and stored frozen as 200- μ L aliquots under nitrogen. A working batch, stored at 0 °C, was used only until there was visible discoloration and then discarded. Iodoacetamide from Sigma was stored at 0 °C, and used without further purification. Tris, citric acid, sodium citrate, acetic acid, ascorbic acid, phosphoric acid, acetonitrile, and TFA were of the highest reagent grade or HPLC grade available.

HPLC

All experiments were done using a Beckman Altex pumping system, with column effluent monitored at 220 nm (Waters Lambda Max detector coupled to Shimadzu integrating recorder). The column used was a Vydac C18 reverse-phase (#218TP54, 5- μ m particle size, 300-Å pore, 4.6 $\times$ 250 mm). Mobile phases were: A, 0.1% TFA in water; B, 0.092% TFA in acetonitrile/water (60% v/v). Peak fractions were collected manually into 1.5-mL polypropylene tubes. With more labile peptides, the tubes were frozen on dry ice. In normal use, peptides were either used immediately or stored in solution at -76 °C or -10 °C. Partially reduced peptides were never concentrated to dryness.

Partial reduction of peptides

TCEP (20 mM) was dissolved in a buffer consisting of 0.17 M citric acid, adjusted to pH 3 with NaOH. This stock solution (TCEP3) can be stored at room temperature with no special precautions and loses little activity over a period of several weeks. Reduction was carried out by mixing equal volumes of TCEP3 and peptide solution, typically in HPLC effluent. Incubation was carried out for various lengths of time, either at room temperature

(22 °C), or at elevated temperature in a thermostatically controlled heating block. In the latter case, both solutions were allowed to equilibrate to temperature for 5–10 min before use to ensure reproducibility.

Rapid alkylation procedure (Gray, 1993)

Iodoacetamide (100 mg) in a 1.5-mL polypropylene tube was dissolved in 200 μ L 0.5 M Tris acetate, pH 8, containing 2 mM EDTA, by heating at 65 °C until a clear solution was obtained with no residual crystals. This supersaturated solution, approx. 2.2 M, was then cooled to room temperature and used within 5–10 min to avoid recrystallization. During reagent preparation, the solution was protected from light to minimize photolytic production of iodine. Peptide solution, 250 μ L of HPLC effluent (pH 2), was drawn into a glass syringe having a fine-tipped needle and then forcibly squirted into the iodoacetamide solution with vigorous mixing. Mixing was discontinued as soon as the peptide solution was added. After 20–30 s, the reaction was quenched by acidification with 400 μ L of 0.5 M citric acid and the mixture was applied directly to the HPLC. The column was washed with a low percentage of buffer B, until the effluent had an absorbance below 0.05, when the gradient was started.

Alkylation with 4-VP

Peptide contained in 0.5 mL of HPLC effluent was alkylated by the addition of 3 μ L of 4-VP, followed by 3 μ L of pyridine. Reaction was carried out in the dark for 20 min at room temperature. After mixing with 1–2 volumes of 0.1% TFA to dilute the acetonitrile, the sample was injected into the HPLC, washed at a low %B until the effluent absorbance was below 0.05, and eluted with a suitable gradient.

Amino acid sequencing

All analyses were carried out by Dr. Robert Schackmann of the Utah Regional Cancer Center, using an ABI Model 477A.

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