# Selective Disulfide Formation in Truncated Apamin and Sarafotoxin†

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ABSTRACT: Apamin and sarafotoxin are small peptide toxins which are 18 and 21 residues long, respectively. They both have cysteines at positions 1, 3, 11, and 15. However, the non-cysteine portions of their sequences and the positions of their disulfides are different. In native apamin, the cysteines form disulfides 1–11 and 3–15, whereas in sarafotoxin they form the 1–15 and 3–11 pairs. Truncated analogs have been synthesized which lack the carboxyl-terminal tails following cysteine-15. When oxidized by glutathione, both truncated sequences retain the ability to selectively populate the disulfide combination observed in the respective full-length parent. This ability is retained in the presence of the denaturing agent 5 M guanidinium chloride. Circular dichroism spectra of the nativelike isomers are nearly identical to those of the parent sequence, and are not affected by heating to 75 °C or exposure to 5 M guanidinium chloride. The $\alpha$ helix observed in apamin is a consequence of both the disulfide topology and the non-cysteine portions of the sequence. There is not much $\alpha$ helix when apamin is forced to adopt the disulfides found in native sarafotoxin or when sarafotoxin is forced to adopt the disulfides found in native apamin.

Apamin and sarafotoxin are small toxins which are 18 and 21 residues long, respectively. They both have cysteines at positions 1, 3, 11, and 15. However, the non-cysteine portions of their sequences are unrelated, and the positions of their disulfides are different. For these peptides, there are three possible isomers containing two intramolecular disulfides, which are globule (1–11, 3–15), ribbon (1–15, 3–11), and beads (1–3, 11–15). They are named after their potential to resemble a spherical globule, an antiparallel ribbon, or a string of two beads. Native apamin contains the globule pairing, whereas sarafotoxin and its homologous endothelin relatives form the ribbon isomer. When fully reduced apamin and sarafotoxin are reoxidized by air, both apamin and sarafotoxin re-form their respective nativelike disulfide combinations in more than 75% of the molecules (Nakajima et al., 1989). Neither peptide forms the beads isomer, probably because its Cys-X-Cys and Cys-X-X-X-Cys loops containing one or three residues between the cysteines are the least favorable sizes for small disulfide loops (Zhang & Snyder, 1989). It is not known which portions of the toxins' sequences aid in discriminating between the sterically allowed globule and ribbon topologies.

The C-terminal tails following cysteine-15 may not be essential for the disulfide discrimination. The circular dichroic (CD)[1] spectrum of truncated globular apamin is similar to the spectrum of the full-length globule isomer (Labbe-Jullie et al., 1991), suggesting that truncation has negligible effects on apamin's secondary structure. NMR determinations of the three-dimensional structure of apamin (Pease & Wemmer, 1988) indicate that residues 16–18 form a frayed $\alpha$ helix extending away from the body of the peptide. Similarly, residues 16–21 in sarafotoxin exhibit no close-range contacts with the disulfide cross-linked core in its NMR-derived structure (Mills et al., 1991).

Disulfide exchange reactions between apamin and mixtures of reduced and oxidized glutathione lead to selection of the globule disulfide combination, both in a physiological buffer and in a denaturing solvent containing 8 M urea (Chau & Nelson, 1992). If this reflects the presence of sequence-specific folding interactions which are resistant to this commonly used denaturing agent, then it would be of interest to define those interactions. If instead the behavior in the denaturing solvent results solely from the placement of cysteines in the linear sequence, then sarafotoxin also should form the globule topology in a denaturing solvent since it has identical cysteine placements.

Experiments described below provide quantitative measurements of the equilibrium constants for forming the globule isomer of truncated apamin and the globule and ribbon isomers of truncated sarafotoxin, where the C-terminal tails following residue 15 have been replaced by a single amide group. The peptides were truncated in an effort to construct minimal length variants which might be capable of selective disulfide formation. Data are reported in glutathione redox buffers, both in 0 M and in 5 M GdmCl.

CD spectra are obtained in a phosphate buffer at 25 °C, pH 7, for the beads, ribbon, and globule isomers of truncated apamin and sarafotoxin, as well as for a flexible derivative containing four blocked cysteine groups. Effects of heating, addition of the denaturant GdmCl, or addition of helix-inducing TFE also are examined.

## MATERIALS AND METHODS

GSH, GSSG, native apamin, and native sarafotoxin b were obtained from Sigma Chemical Co. GSSG was purified by reverse-phase HPLC to remove minor UV-absorbing contaminants.

Synthetic peptides were prepared which correspond to the first 15 residues of apamin and sarafotoxin b. Their sequences are

apamin: C-N-C-K-A-P-E-T-A-L-C-A-R-R-C-am

sarafotoxin b: C-S-C-K-D-M-T-D-K-E-C-L-Y-F-G-am

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[1] Abbreviations: HPLC, high-pressure liquid chromatography; TFA, trifluoroacetic acid; GdmCl, guanidinium chloride; SH4, fully reduced peptide; Scam, peptide with four carboxamidomethylated cysteines, L, globule isomer (disulfides 1–11, 3–15); R, ribbon isomer (1–15, 3–11); B, beads isomer (1–3, 11–15); TFE, trifluoroethanol; NMR, nuclear magnetic resonance; CD, circular dichroism; GSH and GSSG, reduced and oxidized forms of glutathione, respectively; EDTA, ethylenediaminetetraacetic acid; obs, observed.

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These peptides end in an amide (am) group. Methionine-6 in sarafotoxin was replaced by norleucine (M) to minimize heterogeneity attributable to oxidation of the methionine side chains (Harris, 1967). This conservative substitution replaces methionine's sulfur atom by a methylene group.

Peptides were synthesized on a Biosearch 9500 synthesizer using *tert*-butyloxycarbonyl chemistry. 4-Methylbenzhydrylamine resin was used to generate the C-terminal amide group during HF cleavage. The globule, beads, and ribbon isomers of each sequence were constructed in three separate syntheses. Each synthesis employed two 4-methylbenzyl-Cys and two acetamidomethyl-Cys. Disulfides were generated from pairs of cysteines having the same type of sulfur protecting group using previously described protocols (Van Wandelen et al., 1989). These procedures have been used to generate similar sets of isomers in $\alpha$-conotoxin (Nishiuchi & Sakakibara, 1982), full-length apamin (Nakajima et al., 1989), and full-length sarafotoxin b (Aimoto et al., 1990). Each crude isomer was purified by reverse-phase HPLC on a Waters NovaPak 7.8 × 300 mm semipreparative C18 column, employing a linear gradient of increasing acetonitrile in aqueous solutions of 0.1% TFA.

Two additional forms of each sequence were prepared. The fully reduced form (SH4) containing four free SH groups was generated by treating any of the above two-disulfide isomers with excess reduced dithiothreitol at pH 9. The reaction mixture then was acidified to quench disulfide exchange, followed by separation of dithiothreitol and peptides on Millipore SepPak cartridges containing bulk C18 reverse-phase packing material. A second form (Scam) containing four carboxamidomethyl-Cys was prepared by reacting the reduced peptide with excess iodoacetamide and then using SepPak cartridges for peptide isolation.

Amino acid analysis by the precolumn phenylthiocarbamyl derivatization procedure (Bidlingmeyer et al., 1984) confirmed that each of the sarafotoxin and apamin isomers had the correct amino acid composition. Norleucine was included as a quantitative internal standard in the amino acid analyses for purposes of generating molar extinction coefficients for apamin derivatives. At 214 nm, the truncated apamin globule, ribbon, and beads isomers were determined to exhibit $\epsilon$ values of 25 300, 20 300, and 33 400 $M^{-1}$ $cm^{-1}$, respectively. The truncated Scam derivative and native full-length apamin gave larger values of 28 500 and 33 200 $M^{-1}$ $cm^{-1}$, respectively, as expected from the presence of additional amide groups.

Concentrations of stock solutions of reduced apamin were determined by assays of sulfhydryl groups with Ellman's reagent (Riddles et al., 1979). Concentrations of other apamin peptides were determined using the $\epsilon$ values determined above. Sarafotoxin b concentrations were determined by the absorbance at 280 nm, using an $\epsilon$ value of 7250 $M^{-1}$ $cm^{-1}$ for the full-length peptide (Pelton, 1991) and a value of 1200 $M^{-1}$ $cm^{-1}$ for the single tyrosine in the truncated form (Mihalyi, 1968). Stock concentrations of GSH were determined with Ellman's reagent. Concentrations of GSSG were measured using $\epsilon$ = 486 $M^{-1}$ $cm^{-1}$ at 248 nm (Zhang & Snyder, 1988).

Disulfide exchange reactions were performed at pH 7.4 under a nitrogen atmosphere in degassed solvents. Reactions were initiated by combining sulfhydryl-containing molecules dissolved in 1 mM HCl at pH 3 with disulfide-containing molecules dissolved in 100 mM phosphate and 1 mM EDTA at pH 7.4. At selected times, aliquots were withdrawn and adjusted to pH 2 by addition of concentrated HCl. This slows down disulfide exchange by protonating the reactive thiolate anions, generating conditions where negligible disulfide exchange occurs during the time it takes to analyze the quenched aliquots by HPLC (Zhang & Snyder, 1988). Quenched aliquots were analyzed by reverse-phase analytical HPLC on a Waters NovaPak 3.9 × 150 mm C-18 column, using a linear gradient of acetonitrile in aqueous 0.1% TFA for peptide analysis or using 0.1% TFA isocratically for glutathione analysis. The column eluent was monitored by the absorbance at 214 nm.

Equilibrium constants for disulfide exchange reactions were calculated after converting HPLC peak heights to concentrations. Peak heights for GSH and GSSG were analyzed by comparison with injections of stock solutions of known concentrations. For peptides, the best correction factors were obtained by comparing data in the presence and absence of reduced dithiothreitol (Huyghues-Despointes & Nelson, 1992). Dithiothreitol fully reduces the oxidized isomers to the SH4 form. For truncated apamin, the globule and ribbon peaks respectively are 1.2-fold or 1.0-fold taller than peaks generated by equal molar amounts of the reduced peptide. For truncated sarafotoxin b, the globule and ribbon peaks both are 1.3 times taller than the SH4 derivative. Part of these effects may be attributed to broadening of the SH4 peaks, which elute last in the chromatographs.

CD data were obtained on Jasco spectrometers (Models J600 and 500A) employing thermostated 1-mm cells in nitrogen-flushed sample chambers. The spectrometers were calibrated with *d*-10-camphorsulfonic acid (Chen & Yang, 1977). Peptides were dissolved in 20 mM phosphate/0.2 mM EDTA at pH 7.4. Some samples were altered by adding 5 M GdmCl to the phosphate buffer or by adding 50% trifluoroethanol to a buffer in which phosphate was replaced by tris(hydroxymethyl)aminomethane at the same pH. In the GdmCl solutions, absorption of photons by chloride ions prevents acquisition of data below 215 nm. Spectra were acquired at temperatures ranging from 0 to 75 °C.

## RESULTS

*Disulfide Exchange Studies of Truncated Apamin Derivatives.* Each of the three isomers of truncated apamin elutes at a unique position in the chromatogram (Figure 1). To help describe these isomers, the terms "natural" and "non-natural" will sometimes be used to refer to disulfide combinations which match or differ from the combination found in the naturally occurring protein. For truncated apamin, the elution order is natural globule first, followed by ribbon, beads, and then the fully reduced form. For truncated sarafotoxin, the elution order is natural ribbon, globule, beads, and finally SH4. Thus, for both peptide families, the isomer with nativelike disulfides elutes first, and the reduced form elutes last. The natural globule apamin isomer and natural ribbon sarafotoxin isomer best succeed in burying nonpolar groups from interactions with hydrophobic column packings.

In order to prove that equilibrium is reached, the fully reduced apamin form and each of the three oxidized isomers were separately added to buffers containing identical amounts of excess GSH and GSSG. The same final state was achieved, irrespective of peptide starting reagent. This is illustrated in Figure 1, which includes chromatograms of starting peptides before addition of GSH and GSSG and also the final solutions after 3 h of disulfide exchange. Truncated sarafotoxin passes the same type of equilibrium test (data not shown).

Figure 2 depicts the dependence of the equilibrium state of truncated apamin on concentrations of GSH and GSSG. The four solid lines are data obtained from reactions in the absence of GdmCl. There are two major peptide peaks in chromato-

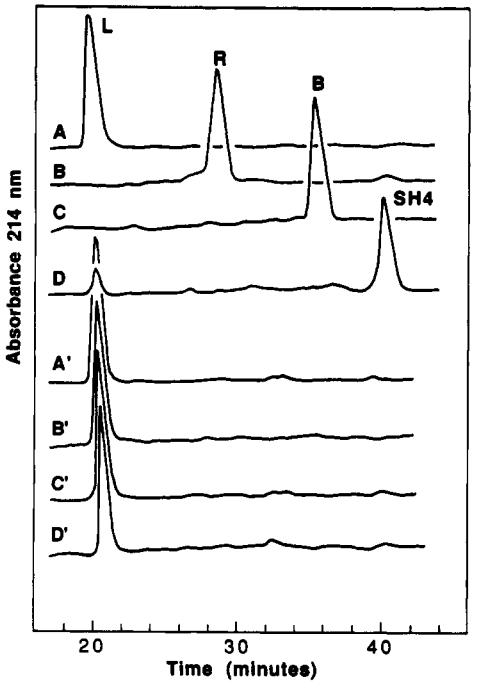


FIGURE 1: Four different starting isomers reach a common final equilibrium state for truncated apamin. [Peptide] = 120 μM, [GSH] = 15.0 mM, [GSSG] = 2.3 mM. (A–D) Reactions at zero time; (A′–D′) reactions after 3 h at pH 7.4, 25 °C. Linear gradient is 12.1–15.0% acetonitrile in 0.1% TFA from 16 to 46 min. Labeled peaks are L (natural globule), R (non-natural ribbon), B (beads), and SH4 (fully reduced).

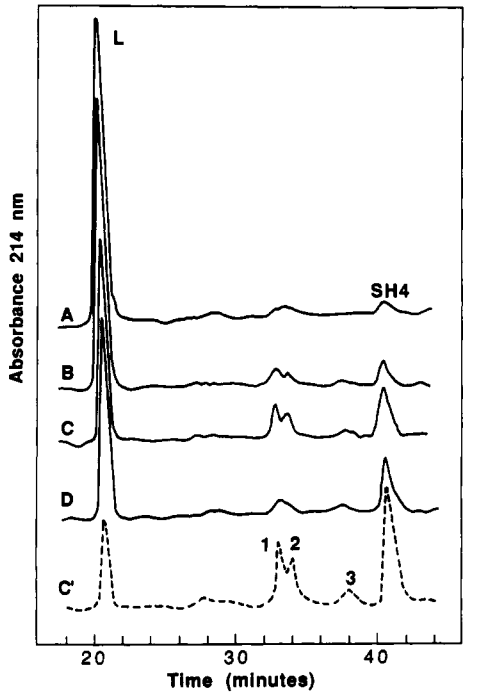


FIGURE 2: Glutathione and GdmCl concentration dependence of the equilibrium state for truncated apamin. Starting isomer = 130 μM natural globule form. Glutathione concentrations (mM GSH/mM GSSG) are (A) 15/2.1, (B) 17/1.9, (C) 22/1.7, (C′) 25/1.7, and (D) 12/0.49. (A–D) Reactions after 3 h in 0 M GdmCl; (C′) 3-h reaction in 5 M GdmCl. Labeled peaks are L (natural globule) and SH4 (fully reduced).

grams A–D, assigned to the L (natural globule) and SH4 (reduced) structures. Results supporting those assignments will be described below. Glutathione peaks elute at much earlier times, and were analyzed in separate injections employing diluted aliquots to bring the large GSH peak on scale. The overall reaction for formation of the monomeric natural globule isomer from fully reduced peptide is given by

$$\text{reduced} + 2\text{GSSG} \rightleftharpoons \text{globule} + 4\text{GSH} \qquad (1)$$

having the equilibrium constant

$$K = \frac{[\text{globule}]}{[\text{reduced}]}\frac{[\text{GSH}]^4}{[\text{GSSG}]^2} \qquad (2)$$

Chromatograms from the four reactions in phosphate buffer are presented in rank order of increasing values for the ratio of $GSH^4/GSSG^2$. From plots A–D, this increasing ratio is accompanied by a decreasing ratio in globule/reduced. The product of these two ratios is a constant. The mean value and standard deviation for measurements of this constant in the four different redox buffers are $K = 0.25 \pm 0.07\ M^2$, as reported in the top of Table I.

Table I: Overall Equilibrium Constants for Formation of Two-Disulfide Isomers from Fully Reduced Truncated Peptides

| protein[a] | structure[b] | $K^c$ ($M^2$) 0 M GdmCl | $K^c$ ($M^2$) 5 M GdmCl |
|---|---|---|---|
| A | L | 0.25 ± 0.07 | 0.09 ± 0.02 |
| S | R | 0.15 ± 0.02 | 0.09 ± 0.02 |
| S | L | 0.021 ± 0.006 | 0.009 ± 0.002 |

[a] A = apamin, S = sarafotoxin. [b] L = globule, R = ribbon. [c] Errors are the standard deviation of the mean for data obtained in six different reaction mixtures.

The assignment of the two main peaks to the natural globule (L) and SH4 forms is based on five types of data. First, addition of synthetic globule or SH4 standard to the quenched reaction aliquot respectively increases the height of the first or last peak of the reaction mixture without broadening those peaks, even when using a very shallow gradient for analysis. Thus, reaction components exactly coelute with genuine samples of globule and SH4 forms. Second, to determine whether the peaks correspond to monomers or dimers, reactions were conducted under conditions where [GSH] and [GSSG] remained constant but total peptide concentration varied by a factor of 3. Equilibrium constants for formation of any dimeric form are proportional to $[\text{peptide}]^2$, as a consequence of the requirement for an intermolecular collision between two monomers. A 3-fold increase in total peptide concentration, under conditions of constant [GSH] and [GSSG], would generate an approximate 9-fold enhancement of dimer peaks at equilibrium. Since peptide concentration had no effect on relative peak heights (data not shown), all peaks in Figure 2 correspond to monomers. Third, assays with Ellman's reagent indicated that there are no free SH groups in the peak eluting at 21 min, so it corresponds to a fully oxidized form. In contrast, the peak eluting at 41 min contains four SH groups per peptide and therefore must be the SH4 form. Fourth, amino acid analysis of the 21-min oxidized form indicated that it lacked the amino acids present in glutathione, so its oxidized cysteines contribute to intramolecular disulfides rather than to mixed disulfides with glutathione. Of the three monomeric intramolecularly oxidized forms (globule, ribbon, and beads), only the natural globule isomer elutes at 21 min. Finally, the ratio of the two major peaks is a function of $[G]^4/[GG]^2$. If one writes down equilibrium constant expressions for all possible relationships between two monomeric forms having different numbers of SH groups, mixed disulfides, and intramolecular disulfides, the only relationships with a $[G]^4/[GG]^2$ dependence are formation of the globule, beads, or ribbon isomers from the SH4 form.

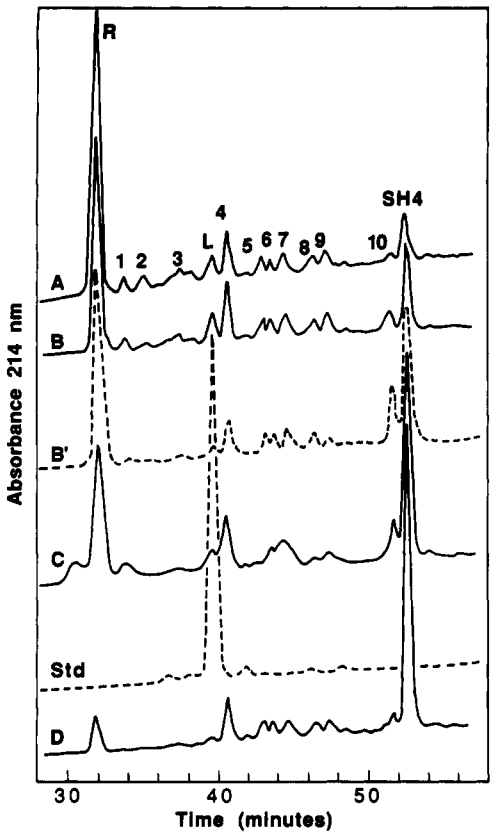


FIGURE 3: Glutathione and GdmCl concentration dependence of the equilibrium state for truncated sarafotoxin. Starting isomer = 45 $\mu$M non-natural globule form. Glutathione concentrations (mM GSH/mM GSSG) are (A) 15/1.4, (B) 16/0.91, (B′) 16/0.74, (C) 13/0.29, and (D) 19/0.31. (A–D) Reactions after 3 h in 0 M GdmCl; (B) 3-h reaction in 5 M GdmCl. Linear gradient is 17.5–37.8% acetonitrile in 0.1% TFA from 28 to 56 min. Std = standard sample of non-natural globule isomer. Labeled peaks are R (natural ribbon), L (non-natural globule), and SH4 (fully reduced).

At equilibrium, truncated apamin is predominantly in the oxidized globule form having the nativelike disulfides, in spite of the fact that the reducing agent GSH exceeds the oxidizing agent GSSG by a 10:1 ratio. In the presence of 5 M GdmCl, the globule isomer is noticeably destabilized. The equilibrium constant for its formation decreases approximately 3-fold from 0.25 $M^2$ in GdmCl's absence to 0.09 $M^2$ in the presence of the denaturant. This latter value still is large enough to populate the natural globule isomer in the strongly reducing buffers. One representative result is included in Figure 2 as a dashed line, C′. It may be compared with plot C in the absence of GdmCl, since both reactions have similar values of [GSH] and [GSSG].

Peaks labeled 1–3 correspond to small amounts of intermediates in the glutathione reaction producing globule from SH4. The intermediates do not coelute with globule, ribbon, beads, or SH4. Their presence and position in the chromatogram resemble the small peaks corresponding to one-disulfide intermediates in studies of full-length apamin (Chau & Nelson, 1992). Their exact structures have not yet been determined.

*Disulfide Exchange Studies of Truncated Sarafotoxin Derivatives.* Figure 3 presents data from corresponding experiments with truncated sarafotoxin. Peaks labeled globule (L), natural ribbon (R), and SH4 were assigned by the same methods described above for apamin, namely, (1) equilibrium dependence on peptide concentration, (2) equilibrium dependence on glutathione concentrations, (3) coelution with standards in shallow gradients, (4) sulfhydryl assays, and (5) amino acid composition analysis. In addition, the equilibrium ratio of globule/ribbon satisfies the requirement that this ratio be constant. The reaction for glutathione-catalyzed intramolecular interconversion of the L and R two-disulfide isomers is given by

$$L(SS)_2 + GSH \rightleftharpoons [(SS)_1(SSG)_1(SH)_1] \rightleftharpoons R(SS)_2 + GSH \quad (3)$$

The species in brackets represents a family of intermediates with one-disulfide loop undergoing intramolecular rearrangements to produce different disulfide pairs. For this reaction:

$$K = \frac{[R][GSH]}{[L][GSH]} = [R]/[L] \quad (4)$$

Thus, the ratio of ribbon (R) to globule (L) isomers has a constant value which is independent of the concentration of GSH or GSSG. The observed value of that ratio is 7:1. For sarafotoxin, numerous intermediates are populated in small amounts.

In Figure 3, the solid lines are arranged according to increasing values for the ratio of $[GSH]^4/[GSSG]^2$. The chromatograms depict equilibria achieved by rearrangements of the relatively unstable non-natural globule isomer, the starting reagent, to the more stable natural ribbon isomer. The same equilibrium state was obtained when starting with the natural ribbon isomer (data not shown). The dashed line B′ in Figure 3 presents data for a reaction mixture containing 5 M GdmCl. GSH and GSSG concentrations in reaction B′ are similar to plot B in which GdmCl was absent. Both the ribbon and globule isomers are destabilized by a factor of 2- or 3-fold in the presence of the denaturing agent. For example, the natural ribbon peak is 2.5 times as tall as the peak for the reduced form in plot B, but is only 1.4 times as tall as the reduced peak in plot B′. This destabilization also appears in the 2-fold difference in values of the equilibrium constants given in the bottom of Table I.

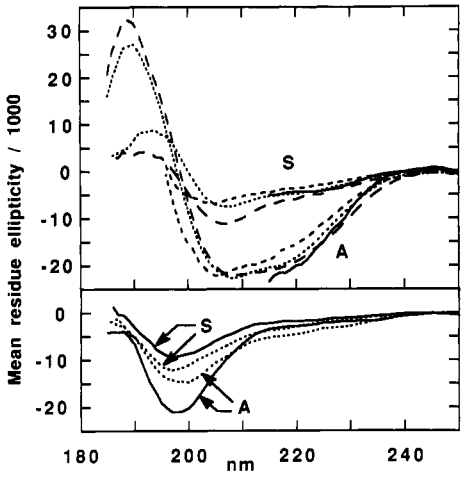


FIGURE 4: Circular dichroism spectra of nativelike and unfolded forms of apamin and sarafotoxin. A, apamin; S, sarafotoxin. All samples at pH 7.4. Mean residue ellipticity calculated in units of degrees centimeter squared per decimole. Upper panel includes spectra for natural globule apamin and natural ribbon sarafotoxin: truncated molecules at 25 °C (···); full-length sequences at 25 °C (– –); truncated molecules at 25 °C, 5 M GdmCl (—); truncated forms heated to 75 °C (– – –). Lower panel gives spectra for unfolded forms: beads (···); Scam (—).

*Circular Dichroism Studies.* The top panel of Figure 4 presents results of experiments investigating the stabilities of molecules with nativelike disulfides. The CD spectrum of full-length native globule apamin exhibits negative dichroism at 222 nm, characteristic of many proteins containing $\alpha$ helix.

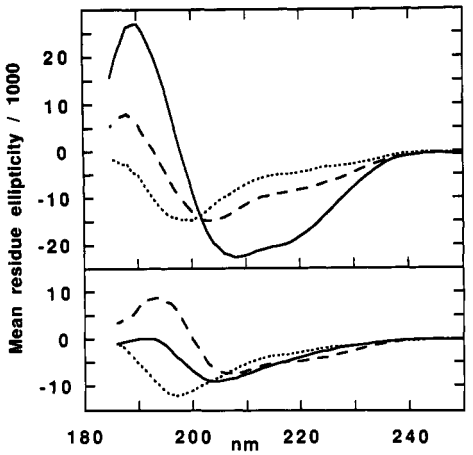


FIGURE 5: Comparison of CD spectra of the three isomers of truncated apamin and sarafotoxin containing two intramolecular disulfide bridges. All samples at pH 7.4, 25 °C. Upper panel for apamin: natural globule (—); ribbon (– –); beads (···). Lower panel for sarafotoxin: natural ribbon (– –); globule (—); beads (···). Other features as in Figure 4.

In contrast, full-length native ribbon sarafotoxin lacks significant dichroism at that wavelength. Truncation of apamin has negligible effects on its spectrum. Neither heating to 75 °C nor addition of 5 M GdmCl substantially affects the spectrum at 222 nm or at lower observable wavelengths. Similarly, the spectrum of full-length native ribbon sarafotoxin is not significantly altered by those treatments.

The lower panel of Figure 4 exhibits spectra for the two derivatives which are least constrained by disulfides. The Scam form lacks disulfides entirely. The beads form contains two small disulfide loops comprised of residues 1–3 and 11–15, separated by an unconstrained region from residues 4 to 10. Both the Scam and beads forms of apamin and sarafotoxin have a negative peak at 198 nm characteristic of the spectra of denatured proteins.

Figure 5 facilitates direct comparison of the spectra of the globule, ribbon, and beads isomers. For apamin, the non-natural ribbon form has substantially less $\alpha$ helix than the natural globule form, as seen by diminished negative dichroism at 222 nm. Its spectrum lies approximately midway between that of the folded natural globule isomer and the relatively unfolded beads isomer. For sarafotoxin, the three spectra exhibit approximately identical small negative dichroism at wavelengths between 205 and 250 nm. At lower wavelengths, the spectrum of the non-natural globule form lies about midway between those of the natural ribbon isomer and the beads isomer, the same pattern observed for the apamin set of isomers.

Figure 6 exhibits the effect of TFE on the Scam form lacking any disulfides. TFE is a solvent which promotes $\alpha$-helix formation (Sonnichsen et al., 1992). In principle, the Scam form is sterically capable of adopting the backbone conformation of the native protein. For both apamin and sarafotoxin, the spectrum of the Scam derivative in the presence of TFE lies between the spectra of the Scam form and the natural isomer in a folding buffer. For these Scam peptides, TFE generates a shift toward more positive dichroism at 198 nm and a corresponding enhancement of negative dichroism at 222 nm usually associated with $\alpha$-helix formation. These changes are greater in absolute magnitude for the apamin sequence.

Figure 7 presents data addressing the question of whether or not the globule or ribbon disulfide topologies are themselves responsible for the conformation observed in native apamin and sarafotoxin, respectively. As seen in the upper panel,

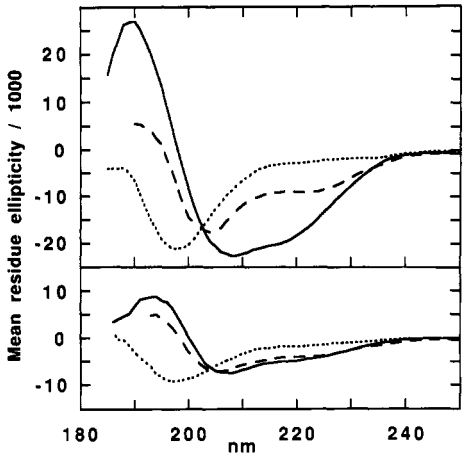


FIGURE 6: Effect of trifluoroethanol on the CD spectra of truncated Scam forms. Upper panel for apamin: natural globule (—); Scam (···); Scam in 50% TFE (– –). Lower panel for sarafotoxin: natural ribbon (—); Scam (···); Scam in 50% TFE (– –). Other features as in Figure 5.

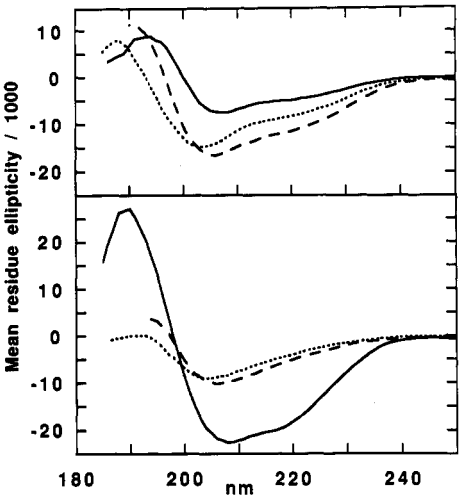


FIGURE 7: Comparison of CD spectra of molecules with different sequences but identical disulfide pairs. Upper panel for ribbon isomers: natural sarafotoxin (—): non-natural apamin (···); non-natural apamin in 50% TFE (– –). Lower panel for globule isomers: natural apamin (—); non-natural sarafotoxin (···); non-natural sarafotoxin in 50% TFE (– –). Other features as in Figure 5.

when the apamin sequence is forced to adopt the ribbon disulfide pairs found in native sarafotoxin, the non-natural apamin ribbon spectrum is somewhat different from that of natural ribbon sarafotoxin. Non-natural ribbon apamin exhibits greater negative dichroism at both 198 and 222 nm compared with its sarafotoxin counterpart. Addition of TFE appears to slightly enhance $\alpha$-helix formation in the non-natural apamin ribbon isomer, as seen by a shift toward more positive dichroism at 198 nm and more negative dichroism at 222 nm. The lower panel indictes that when the sarafotoxin sequence is forced to adopt the globule disulfide pairs found in native apamin, the non-natural sarafotoxin globule form does not acquire the negative dichroism at 222 nm displayed by natural globule apamin. Moreover, TFE has very little effect on the spectrum of non-natural globule sarafotoxin.

## DISCUSSION

*Similarities between Truncated and Full-Length Sequences.* In order to determine which regions of the apamin and sarafotoxin sequences are responsible for stabilization of the natural topologies, sequences were truncated at cysteine-15.

These provide the shortest segments potentially capable of retaining selective disulfide formation.

Results presented above suggest that truncation of apamin or sarafotoxin does not affect the secondary structure or the relative stabilities of the three topological isomers of each sequence. Circular dichroism spectra are similar for truncated and full-length native variants. This was already known for apamin (Labbe-Jullie et al., 1991), but is new information for sarafotoxin. The results are not surprising, since the NMR-derived structures of apamin (Pease & Wemmer, 1988) and sarafotoxin b (Mills et al., 1991) do not indicate packing of the C-terminal tail against the rest of the molecule. The values of the overall disulfide exchange equilibrium constant for truncated apamin in 0 and 5 M GdmCl (0.25 and 0.09 $M^2$, respectively) are similar to values previously reported (Chau & Nelson, 1992) for full-length apamin in 0 and 8 M urea (0.42 and 0.09 $M^2$, respectively). Equilibrium constants for glutathione reactions with full-length sarafotoxin are not available for comparison with the values described here for the truncated sequence. However, the relative populations of ribbon/globule/beads for truncated sarafotoxin in glutathione reactions, 7:1:0, are similar to the 3:1:0 proportions produced by results in air oxidation of full-length sarafotoxin (Nakajima et al., 1989).

*Disulfide Exchange Reactions in Truncated Sarafotoxin.* Results in Table I are the first data reported for disulfide exchange equilibria in the endothelin/sarafotoxin family. Data reveal that sarafotoxin has the same characteristics previously described for apamin (Chau & Nelson, 1992), namely, (1) a strong tendency to produce the oxidized isomer with nativelike disulfides, even in a reducing solvent, and (2) persistence of that tendency in the presence of high concentrations of a denaturing agent.

Steps for formation of the sarafotoxin ribbon isomer may be represented by

$$\text{step 1: } P(SH)_4 + GSSG \rightleftharpoons P(SS)_1(SH)_2 + 2GSH \quad (5)$$

$$\text{step 2: } P(SS)_1(SH)_2 + GSSG \rightleftharpoons R(SS)_2 + 2GSH \quad (6)$$

$$\text{step 3: } R \rightleftharpoons R' \quad (7)$$

where P represents the peptide chain at early stages in the reaction and R is the ribbon isomer. The first two steps are overall reactions for formation of the first and second intramolecular disulfides, respectively. The third step considers a possible conformational change occurring after formation of the second intramolecular disulfide. R and R′ both have the ribbon disulfide topology. Equilibrium constants for these steps will be labeled $K_1$–$K_3$, respectively, in discussions below.

The absolute values of the equilibrium constants in Table I may be compared with data for glutathione reaction with single small disulfide loops differing in the number and types of amino acids in the intervening sequence between the paired cysteines (Zhang & Snyder, 1989). In 5 M GdmCl, overall equilibrium constants for glutathione generation of a single small peptide loop have values ranging from 0.0023 to 0.10 M, depending primarily on loop size. Larger loops exhibit smaller values in a denaturing solvent (Snyder, 1987), given the diminished probability for collision between the two cysteines. Thus, the maximum value of $K_{overall}$ for consecutive formation of two independent loops in an unstructured peptide is about (0.1 M) × (0.1 M) = 0.010 $M^2$.

For conotoxin, a small peptide with four cysteines, the reaction for formation of its native globule isomer by glutathione exhibits an equilibrium constant of 0.009 $M^2$ (Zhang & Snyder, 1991). Compared with the value of 0.010 $M^2$ described above, conotoxin therefore does not demonstrate much difference from behavior expected for an unfolded peptide. In contrast, both sarafotoxin and apamin have $K$'s which respectively are 15- and 25-fold enhanced relative to the 0.010 $M^2$ value.

These equilibrium constants are a function of the relative thermodynamic stabilities of the structureless-beginning SH4 form with no disulfides and the final structured native conformation having two disulfides. Discussion of the large value of $K$ observed in full-length apamin described this behavior as resulting from positive cooperativity (Chau & Nelson, 1992). That discussion proposed a reasonable kinetic mechanism suggesting that formation of the first disulfide in apamin might induce folding which juxtaposes the remaining two cysteines, leading to enhancement of the rate constant for the chemical formation of the second disulfide bond. In this case, $K_{overall} = K_1K_2$ and is large because $K_2$ is significantly larger than values observed in studies of single disulfide loops. Here, folding occurs prior to formation of the second disulfide.

For purposes of stimulating future kinetic experiments, it is useful to suggest an alternative explanation. Suppose formation of the second disulfide is necessary to nucleate folding of one turn of helix comprising the previously described "disulfide-stabilized helix motif" (Tamaoki et al., 1991) and this motif then propagates into a longer helixlike region. Here, folding occurs after formation of the second disulfide instead of before that formation. $K_{obs}$, the constant generated from ratios of HPLC peak heights, then is a function of $K_1$, $K_2$, and $K_3$, with $K_3$ having a value much greater than 1. In this case, the HPLC peak labeled "R" actually represents the sum of unfolded R molecules and folded R′ molecules present at the moment of acidification of the reaction aliquot. Both sets of molecules would adopt a single common conformation while waiting to be injected into the HPLC column. Since $K_3 = [R']/[R]$, it follows that $[R'] = [R]K_3$. Hence

$$[\text{peak "R"}] = [R]_{total} = [R] + [R'] = [R] + [R]K_3 = R(1 + K_3) \quad (8)$$

Then

$$K_{obs} = \frac{[R]_{total}[GSH]^4}{[SH4][GSSG]^2} = K_1K_2(1 + K_3) \quad (9)$$

The observed constant would be large compared with values expected for unstructured peptides because a conformational equilibrium, step 3, pulls the reaction to the right after both nativelike disulfides are present.

For either mechanism, the enhancement of $K_{obs}$ in a denaturing solvent is unexpected. In such solvents, one would not anticipate that two cysteines are specially juxtaposed in a one-disulfide intermediate to generate a large value of $K_2$, or that folding occurs in an isomer containing two disulfides to generate a large value of $K_3$.

*Circular Dichroism of Ribbon and Globule Isomers.* CD spectra of full-length natural globule apamin and full-length natural ribbon sarafotoxin have been published previously, as well as spectra of unfolded derivatives lacking any disulfides (Huygheus-Despointes & Nelson, 1992; Labbe-Jullie et al., 1991; Tamaoki et al., 1992). Data presented above for non-natural ribbon apamin, non-natural globule sarafotoxin, and the beads isomers of both sequences provide new information.

The only derivatives which exhibit strong $\alpha$-helical content, demonstrated by significant negative dichroism at 222 nm, are the natural globule isomers of truncated or full-length

apamin. The globule disulfide combination alone is not sufficient to stabilize the helix, since non-natural globule sarafotoxin lacks the characteristic spectral features. Results described above for samples dissolved in 50% TFE suggest that apamin's sequence has a greater propensity to form $\alpha$ helix than sarafotoxin. However, the apamin sequence alone is not sufficient, since non-natural ribbon apamin lacks a large negative shoulder at 222 nm, even in the presence of 50% TFE. In principle, the ribbon topology having an antiparallel alignment of the 1–3 and 11–15 segments requiring a single chain reversal could tolerate a longer C-terminal helix than the parallel alignment of the globule topology requiring two chain reversals. Yet it is the latter isomer in which the $\alpha$ helix is best stabilized. The disulfide-stabilized $\alpha$-helix motif in truncated apamin is very stable, resisting denaturation by 5 M GdmCl or heating to 75 °C.

Spectra of natural ribbon and non-natural globule sarafotoxin are generally similar to each other (Figure 5). These forms cannot have similar conformations, given contrasting parallel and antiparallel alignments of the 1–3 and 11–15 segments. Thus, the spectral shape exhibited by these molecules is not indicative of a particular conformation. The absence of substantial negative dichroism at 198 nm, suggesting the absence of a random assortment of conformations, does not necessarily imply that non-natural globule sarafotoxin adopts a unique stable folded conformation. Small unfolded molecules dominated by a nested pair of small disulfide loops restricting peptide mobility probably cannot adopt the same mixture of conformations available to unfolded molecules lacking such loops. Thus, for these derivatives, CD data are not very helpful for evaluating the stability of the natural ribbon sarafotoxin form or addressing the question of whether non-natural ribbon apamin adopts the conformation in natural ribbon sarafotoxin.

*Conclusions.* Since the truncated molecules are competent to direct formation of specific but different disulfides, these families will provide a nice system for future investigations of which of the 11 non-cysteine residues in the truncated molecules generate disulfide-pairing specificity. These molecules probably constitute the smallest peptides which have been demonstrated to have sufficient information content to distinguish between two alternative folded conformations. Use of a norleucine in sarafotoxin and deletion of the C-terminal tails of these proteins remove several types of reactive amino acids, namely, methionine, histidine, and tryptophan, which otherwise might contribute to difficulties during peptide synthesis and deprotection (Stewart & Young, 1984). Furthermore, removal of the nonpolar Val-Ile-Trp sequence comprising residues 19–21 of sarafotoxin probably helps to increase solubility. These chemical and physical properties further enhance the utility of the truncated sequences for future work.

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