Research Article

Effects of Selective Substitution of Cysteine Residues on the Conformational Properties of Chlorotoxin Explored by Molecular Dynamics Simulations

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Abstract: Chlorotoxin (CTX) is a 36-amino acid peptide with 8 Cys residues that forms four Cys-Cys bonds. It has high affinity for the glioma-specific chloride channel and matrix metalloprotease-2. Structural and binding properties of CTX analogs with various Cys residue substitutions with L-α-aminobutyric acid (Abu) have been previously reported. Using 4.2 μs molecular dynamics, we compared the conformational and essential space sampling of CTX and analogs [Abu/Ser2,19]CTX, [Abu/Ser2,19]CTX, [Abu/Ser2,19]CTX, [Abu/Ser2,19]CTX, and [Abu/Ser2,19]CTX. The native and substituted peptides maintained a high degree of α-helix propensity from residues 8 through 21, with the exception of [Ser5-20]CTX and [Abu16,33]CTX. In agreement with previous circular dichroism spectropolarimetry results, the C-terminal β-sheet content varied less from residues 25 through 29 and 32 through 36 and was well conserved in all analogs, except [Abu16,33]CTX, [Ser20,35]CTX, [Abu2,16,19,20,28,33,35]CTX, and [Ser2,16,19,20,28,33,35]CTX. The Cys16-Cys33 and Cys25-Cys35 Cys-Cys bonds appear to be required to maintain the αβ-motif of CTX. Their selective substitution with Ser16,33 however, may mitigate the destabilizing effect of Cys16-Cys33 substitution by the formation of an inter residue H-bond from OγH of Ser16 to OγH of Ser33 bridged by a water molecule. All peptides shared considerable sampled conformational space, which explains the retained receptor binding of the nonnative analogs.

Keywords: αβ motif; Abu; chlorotoxin; Cys; disulfide bond; insectotoxin; isosteric substitution; L-α-aminobutyric acid; molecular dynamics; Ser

1. Introduction

Chlorotoxin (CTX) is a peptide toxin in the venom of the deathstalker scorpion (Leiurus quinquestriatus) [1,2]. The peptide binds with high affinity to chloride channels, causing paralysis in invertebrates, but it has minimal to no effect on vertebrates or mammals [2]. Because of its high affinity and selectivity, CTX was originally used as a tool to characterize the function of chloride channels in electrophysiology experiments. Pharmacologic interest in the peptide increased after CTX was shown to bind with high affinity to glioma-specific chloride channels on the surface of high-grade intrinsic brain tumors (glioblastoma multiforme), as well as other tumors of neuroectodermal embryologic origin [3-5]. CTX has been considered a potential lead for the
development of novel therapeutic agents, imaging adjuncts, and intraoperative optical imaging
“tumor dyes/paints” [6-10].

CTX consists of 36 amino acids with 8 Cys residues at positions 2, 5, 16, 19, 20, 28, 33, and 35 (I-VIII for homology modeling) that form 4 Cys-Cys bonds: 2-19, 5-28, 16-33, and 20-35 (I-IV, II-VI, III-VII, and V-VIII) (Figure 1). The resulting secondary and tertiary structure are known as an αβ
(βαββ) motif, a folding scaffold common to insectotoxins, insect defensins, plant γ-thionins, and inhibitory cystine knot peptides [11-20]. Multisequence alignment of 20 scorpion toxin–derived peptides shows that they share 49% to 88% sequence similarity [11-15]. Overlays of the 3-dimensional structures of several of these peptides have root-mean-square deviation (RMSD) less than 0.1 nm, which indicates the stability of the αβ motif [21]. The observed differences in channel blocking and molecular selectivity of the toxins are due to subtle differences in residue charge and sidechain conformation that affect surface electrostatic charge distribution and complementarity [21].

Figure 1. Amino acid sequence of chlorotoxin, demonstrating the Cys-Cys bond topology between Cys²-Cys¹⁹, Cys²-Cys²⁸, Cys¹⁶-Cys³⁵, and Cys²⁰-Cys³⁵, shown as yellow with black brackets representing disulfide bonds, and homology numbering shown as Roman numerals. The conserved secondary structural motifs of an α-helix from Ala¹³ to Cys²⁰, a β-sheet from Cys² to Cys⁵, and 2 antiparallel β-sheets from Lys²⁷ to Tyr²⁸ and Lys³² to Leu³⁴ are shown as a blue cylinder and red arrows, respectively.

The locations of the Cys-Cys bonds within this family of peptides are important for maintaining secondary and tertiary structural features and providing a degree of protease resistance [16,22-26]. Despite the evolutionarily conserved Cys-Cys bonds in this family of insectotoxins, the ability to selectively remove some of these bonds is well documented [24-28]. Selective substitution of Cys residues with L-α-aminobutyric acid (Abu) in the charybdotoxin and leiurotoxin I peptides demonstrated successful substitution of the Cys¹⁶-Cys³¹ disulfide bond without significant effects on oxidative folding [24-28]. Previous work by Ojeda et al. demonstrated that selective substitution of the Cys²-Cys¹⁹ or Cys²-Cys²⁸ bonds of CTX with Abu residues had little effect on peptide conformation, whereas the Cys¹⁶-Cys³¹ and Cys²⁰-Cys³⁵ disulfide bonds were critical to the process of oxidative folding and obtaining nativelike peptides [29]. The authors also demonstrated that complete substitution of all disulfide bonds resulted in a peptide that maintained its biological activity, but with complete disruption of its secondary and tertiary conformation and significantly increased susceptibility to serum proteases [29].

The tertiary structure of CTX in water (Protein Data Bank ID: 1CHL) has been determined using high-field ¹H nuclear magnetic resonance spectroscopy (NMR) (Figure 2) [30]. The presence of the N-terminal β-sheet from Cys² to Cys⁵ is variable and dependent on the algorithm used to determine the structure [31,32]. In the current study, we used molecular dynamics (MD) simulations of the structures of CTX and Abu- and Ser-substituted CTX analogs to investigate the role of the Cys-Cys disulfide bonds in stabilizing the αβ motif. Emphasis was placed on investigating the role of hydrophobic (Abu) versus hydrophilic (Ser) isosteric substitutions, the distribution of the hydration shell around each respective residue substitution, and subsequent changes in secondary and tertiary conformation.
Figure 2. Solution conformation of chlorotoxin (CTX) as determined by $^1$H nuclear magnetic resonance spectroscopy (NMR) (Protein Data Bank ID: 1CHL) demonstrating the $\alpha$-helix (Ala$^{13}$ to Cys$^{20}$) and 2 antiparallel $\beta$-sheets (Lys$^{27}$ to Tyr$^{29}$ and Lys$^{32}$ to Leu$^{34}$) with the 4 Cys-Cys bonds: Cys$^{2}$-Cys$^{19}$, Cys$^{5}$-Cys$^{28}$, Cys$^{16}$-Cys$^{33}$, and Cys$^{20}$-Cys$^{35}$, shown in yellow. The N-terminal $\beta$-sheet from Cys$^{2}$ to Cys$^{5}$ is not present in the lowest energy NMR conformation. Location of the Cys-Cys substitutions with L-$\alpha$-aminobutyric acid (Abu) or Ser (not shown) are in turquoise with the associated naming conventions: [Abu/Ser$^{2,19}$]CTX, [Abu/Ser$^{5,28}$]CTX, [Abu/Ser$^{16,33}$]CTX, [Abu/Ser$^{20,35}$]CTX, and [Abu/Ser$^{2,5,16,19,20,28,33,35}$]CTX.

2. Results

Details outlining the parameterization of the Abu residue are provided in Appendix A.

2.1 Biophysical Properties

The mean ± SD: $R_g$, RMSD$_{CTX}$, RMSD$_{AVG}$, fraction of sampled secondary structure ($\alpha$-helix, $\beta$-sheet, and $\beta$-bend/turn), SASA, SASA$_{Hydrophobic}$, and SASA$_{Hydrophilic}$ values for each peptide are shown in Table 1, along with their tests of data homoscedasticity (Levene’s test) and the associated Welch’s ANOVA. The post hoc analysis results are shown in Table B1. Selective substitution of the Cys residues with either Ser or Abu caused small increases in the $R_g$, with the exception of [Ser$^{2,19}$]CTX, which was similar to the native peptide, and [Ser$^{20,35}$]CTX, which was more compact. Complete substitution of the Cys residues with either Ser or Abu resulted in significant increases in the $R_g$, with [Ser$^{2,5,16,19,20,28,33,35}$]CTX being less compact than [Abu$^{2,5,16,19,20,28,33,35}$]CTX. There were significant differences in the $R_g$ values of all peptides, with the exception of 9 pairwise comparisons for RMSD$_{CTX}$ and 1 pairwise comparison for RMSD$_{AVG}$ (Table B1).
Table 1. Calculated biophysical properties with ANOVA

| Peptide          | \( R_g \), nm  | RMSD\(_{CTX} \), nm\(^a\) | RMSD\(_{AVG} \), nm\(^b\) | \( \alpha \)-helix, \( \rho \) | \( \beta \)-sheet, \( \rho \) | Bend/turn, \( \rho \) | SASA, nm\(^2\) | SASA\(_{hydrophobic} \), nm\(^2\) | SASA\(_{hydrophilic} \), nm\(^2\) |
|------------------|----------------|--------------------------|--------------------------|----------------|----------------|----------------|-------------|----------------|----------------|
| CTX              | 0.88 ± 0.02    | 0.73 ± 0.02               | 0.73 ± 0.02               | 0.31 ± 0.05   | 0.16 ± 0.02   | 0.17 ± 0.05   | 29.4 ± 1.3  | 13.9 ± 1.1     | 15.5 ± 1.3     |
| [Abu\(^2\),\(^{19}\)]CTX | 0.94 ± 0.03    | 0.71 ± 0.02               | 0.74 ± 0.02               | 0.39 ± 0.09   | 0.14 ± 0.05   | 0.11 ± 0.09   | 30.2 ± 1.5  | 11.0 ± 0.8     | 19.2 ± 1.1     |
| [Ser\(^2\),\(^{19}\)]CTX | 0.88 ± 0.02    | 0.73 ± 0.02               | 0.73 ± 0.02               | 0.27 ± 0.04   | 0.20 ± 0.03   | 0.26 ± 0.02   | 27.9 ± 1.0  | 9.8 ± 0.7      | 18.1 ± 0.7     |
| [Ser\(^5\),\(^{28}\)]CTX | 0.96 ± 0.04    | 0.73 ± 0.02               | 0.74 ± 0.02               | 0.34 ± 0.08   | 0.16 ± 0.08   | 0.08 ± 0.07   | 30.9 ± 1.4  | 12.6 ± 0.8     | 18.3 ± 1.2     |
| [Abu\(^5\),\(^{33}\)]CTX | 0.94 ± 0.04    | 0.73 ± 0.02               | 0.76 ± 0.02               | 0.31 ± 0.07   | 0.19 ± 0.06   | 0.13 ± 0.14   | 30.1 ± 1.5  | 11.8 ± 0.9     | 18.3 ± 1.1     |
| [Ser\(^5\),\(^{33}\)]CTX | 0.90 ± 0.04    | 0.73 ± 0.02               | 0.72 ± 0.02               | 0.29 ± 0.06   | 0.13 ± 0.06   | 0.22 ± 0.08   | 28.7 ± 1.9  | 11.4 ± 0.9     | 17.3 ± 1.3     |
| [Abu\(^{2},\(^{16},\(^{33}\)]CTX | 0.91 ± 0.03    | 0.73 ± 0.02               | 0.72 ± 0.02               | 0.32 ± 0.08   | 0.16 ± 0.04   | 0.19 ± 0.07   | 29.8 ± 1.5  | 10.8 ± 1.1     | 19.0 ± 1.0     |
| [Ser\(^{2},\(^{16},\(^{33}\)]CTX | 0.86 ± 0.01    | 0.72 ± 0.02               | 0.72 ± 0.02               | 0.30 ± 0.06   | 0.14 ± 0.04   | 0.27 ± 0.06   | 27.0 ± 0.9  | 9.7 ± 0.5      | 17.3 ± 0.8     |
| [Abu\(^{2},\(^{5},\(^{16},\(^{19},\(^{20},\(^{28},\(^{33},\(^{35}\)]CTX | 1.07 ± 0.15    | 0.72 ± 0.02               | 0.61 ± 0.02               | 0.28 ± 0.09   | 0.03 ± 0.05   | 0.21 ± 0.14   | 33.4 ± 3.2  | 9.9 ± 1.6      | 23.5 ± 2.1     |
| [Ser\(^{2},\(^{5},\(^{16},\(^{19},\(^{20},\(^{28},\(^{33},\(^{35}\)]CTX | 1.18 ± 0.20    | 0.73 ± 0.02               | 0.65 ± 0.02               | 0.30 ± 0.09   | 0.05 ± 0.06   | 0.09 ± 0.10   | 35.7 ± 3.3  | 10.7 ± 1.5     | 25.0 ± 2.5     |
| Levene’s P Value | <.001          | <.001                     | <.001                     | <.001          | <.001          | <.001          | <.001       | <.001          | <.001          |
| ANOVA F-Statistic | 7,833.2        | 636.2                     | 15,343                    | 1,371.8        | 4,616.6        | 6,629.6        | 6,077.9     | 8,666.5        | 8,877.4        |
| F (df1, df2)      | (10, 17,326)   | (10, 17,588)              | (10, 17,543)              | (10, 17,447)   | (10, 17,311)   | (10, 17,537)   | (10, 17,520) | (10, 17,537)   |
| ANOVA P value     | <.001          | <.001                     | <.001                     | <.001          | <.001          | <.001          | <.001       | <.001          | <.001          |

Abbreviations: Abu, L-\( \alpha \)-aminobutyric acid; ANOVA, analysis of variance; CTX, chlorotoxin; df1, degrees of freedom between the groups; df2, degrees of freedom within the groups; \( R_g \), radius of gyration; RMSD, root-mean-square deviation; SASA, solvent-accessible surface area.

\(^a\) \( \alpha \)-trace comparison between the peptide and the average conformation of CTX.

\(^b\) \( \alpha \)-trace comparison between the peptide and the average conformation of itself.
The fraction of α-helix from residues 2 through 9, 12 through 13, 17 through 20, and 22 through 24 was higher for [Abu²,19]CTX, [Ser²,19]CTX, and [Ser²,19]CTX. The peptides with the highest SASA, respectively, had the lowest SASA, whereas [Ser²,19]CTX, [Ser²,19]CTX, and [Ser²,19]CTX had the highest values. The peptide with the lowest SASA, Hydrophobic, and SASA, Hydrophilic, values showed significant differences, with the exception of several pairwise comparisons (Table B1).

2.2 Secondary Structure

The per-residue fractions of sampled secondary structure for each of the simulated peptides are shown in Figure 3. The secondary structure for CTX was consistent with its 1H NMR solution conformation, having 3 prominent β-turns/bends in the N-terminal domain from residues 6 through 9 and 12 through 13, α-helix from residues 15 through 20, and 2 antiparallel β-sheets from residues 27 through 29 and 32 through 34, with the expected intervening β-turns/bends from residues 21 through 24 and 30 through 31. Except for [Abu²,19]CTX and [Ser²,19]CTX, all other peptides maintained a high degree of secondary structure retention during simulations.

2.3 Structural Flexibility

The per-residue Ca-trace RMSFs comparing each of the simulated peptides with the average conformation of CTX and the average conformation of themselves are shown in Figure 3 and Figure B1. There were only minor differences in the per-residue RMSF between CTX and that of the average conformation. The most flexible regions of CTX correspond to the turn/bend regions from residues 8 through 9, 12 through 13, 21 through 24, and 30 through 31. [Abu²,19]CTX showed increased flexibility from residues 6 through 14, which corresponded to lengthening and destabilization of the α-helix region. [Ser²,19]CTX, [Abu²,28]CTX, and [Ser²,28]CTX were more stable on a per-residue RMSF than CTX and all other peptides. [Ser²,28]CTX had increased flexibility from residues 1 through 14, which corresponded to disruption of the α-helical region and the development of 2 N-terminal antiparallel β-sheets. There was also a slight increase in the flexibility of the C-terminal region from residues 22 through 32, which corresponded to a slight decrease in stability of the 2 antiparallel β-sheets. The degree of Ca-trace RMSF for [Abu²,19]CTX, [Ser²,19]CTX, and [Abu²,28]CTX was similar to CTX. [Abu²,28]CTX and [Ser²,28]CTX demonstrated significant flexibility throughout the peptide, as would be expected based on the significant disruption of peptide secondary structure that occurs with complete removal of all Cys-Cys disulfide bonds.
Figure 3. Graphs showing root-mean-square fluctuation from the time average conformation of CTX (RMSF, black) and fraction of sampled secondary structure (α-helix, blue; β-sheet, red; and β-turn/bend/loop, green) as a function of residue number for all 11 chlorotoxin (CTX) peptides.

2.4 Interactions

The per-residue H-bond donor/acceptor populations are shown in Figure B2. Residues with the highest fraction of H-bond donor-acceptor participation were predominantly in the N-terminal region, especially residues 8 through 20, which have the greatest propensity for α-helical formation. This pattern held for CTX, [Abu2,19]CTX, [Ser2,19]CTX, [Ser5,28]CTX, [Abu16,33]CTX, and [Abu20,35]CTX. [Abu5,20]CTX had a donor-acceptor peak from residues 23 through 25. This corresponds to elongation of the α-helical region and a β-turn from residues 22 through 24. [Ser16,33]CTX, [Ser20,35]CTX, and [Ser2,5,16,19,20,28,33,35]CTX showed significant disruption of the N-terminus through...
the α-helical H-bonding pattern with more uniform values throughout the residues.

[Abu\textsuperscript{2,5,16,19,20,28,33,35}]CTX had peaks corresponding to the α-helical region and residues 25 and 29, which represent β-turn formation.

Per-residue relative SASA (rSASA) and ΔrSASA are shown in Figure B3. The most solvent-shielded residues for CTX were the Cys residues 5, 16, 19, 20, 28, and 33. The N-terminal and C-terminal Cys residues were significantly less solvent shielded. The central 16, 20, 28, and 33 residues are the most solvent shielded in the native peptide and all of the single Cys-Cys disulfide bond substituted peptides. In general, Abu substitution tends to favor solvent shielding, whereas the use of Ser tends to favor solvent exposure. Noted exceptions are [Abu\textsuperscript{2,5,16,19,20,28,33,35}]CTX and [Ser\textsuperscript{2,5,16,19,20,28,33,35}]CTX, in which residues at the substituted positions experienced the greatest degree of decreased rSASA.

The BB-BB and SC-SC contact probability maps are shown in Figure 4. For CTX, most of the high-probability contacts are SC-SC, with the expected long-distance contacts between the 2,19; 5,28; 16,33; or 20,35 disulfide bonded residues and the contacts between the N-terminal residues with the α-helix from residues 15 through 20 and the 2 C-terminal β-sheets from residues 27 through 29 and 32 through 34. The only long distance BB-BB contacts are those expected between the 2 antiparallel β-sheets and interactions between residues 4 and 5 with 31 through 33.

[Abu\textsuperscript{2,35}]CTX allowed for an increase in both SC-SC and BB-BB contacts. The removal of this Cys disulfide bond allowed for increased interactions between the N-terminal region, the α-helix, and the C-terminal antiparallel β-sheets. There were also increased BB-BB interactions between the α-helix from residues 12 through 17 and the proximal β-sheet from residues 24 through 28 and between the N-terminal 8 residues and residues 28 through 32. The contact probability map for [Ser\textsuperscript{2,35}]CTX was much more similar to the native peptide for both SC-SC and BB-BB interactions.

[Abu\textsuperscript{5,29}]CTX contact probabilities were very similar to those of CTX. [Ser\textsuperscript{5,29}]CTX was more similar to [Abu\textsuperscript{5,29}]CTX except that the interaction between the N-terminal residues and α-helix with the proximal β-sheet were much less pronounced and an interaction was present between the N-terminal residues 1 through 8 and the α-helix from residues 13 through 19. The [Abu\textsuperscript{16,33}]CTX and [Ser\textsuperscript{16,33}]CTX contact probability maps were similar and shared substantial similarity with that of [Abu\textsuperscript{5,29}]CTX. [Abu\textsuperscript{20,35}]CTX and [Ser\textsuperscript{20,35}]CTX were more similar to native CTX. [Abu\textsuperscript{5,16,19,20,28,33,35}]CTX showed almost no SC-SC interactions, with low-frequency BB-BB interactions between multiple residues. [Ser\textsuperscript{2,5,16,19,20,28,33,35}]CTX is the exact opposite, with few, if any, BB-BB interactions and sparsely populated SC-SC interactions.

Calculated CβHβ-to-CβHβ center-of-mass distances and probability of proximity within 0.26 nm are shown in Table 2, along with their tests of data homoscedasticity (Levene’s test) and the associated Welch’s ANOVA. The post hoc analysis results are shown in Table B3. The removal of a Cys-Cys disulfide bond in most of the substituted peptides resulted in significant increases in the CβHβ-to-CβHβ center-of-mass distances and decreases in the probabilities of contact, with the exception of [Ser\textsuperscript{16,33}]CTX and [Abu\textsuperscript{20,35}]CTX. The post hoc analysis revealed only minor variations in interaction distances, other than as noted above.
Figure 4. Contact probability maps for backbone-backbone contacts (below the diagonal) and sidechain-sidechain contacts (above the diagonal) as a function of residue number for all 11 chlorotoxin (CTX) peptides. Color scale shows the relative probability of contact.
Table 2. Calculated CβHβ-to-CβHβ center-of-mass distances and probability of proximity ≤0.26 nm for native CTX and Abu- and Ser- substituted peptides\textsuperscript{a,b}.

| Peptide          | Mean±SD, nm (Probability of Contact) |
|------------------|--------------------------------------|
|                  | 2-19       | 5-28       | 16-33      | 20-35      |
| CTX              | 0.24±0.01  | 0.21±0.01  | 0.26±0.01  | 0.21±0.01  |
|                  | (0.98)     | (0.99)     | (0.71)     | (1.00)     |
| [Abu\textsuperscript{2,19}]CTX | 0.45±0.08  | 0.22±0.02  | 0.21±0.01  | 0.21±0.01  |
|                  | (0.02)     | (1.00)     | (1.00)     | (1.00)     |
| [Ser\textsuperscript{2,19}]CTX | 0.43±0.04  | 0.22±0.02  | 0.21±0.01  | 0.21±0.01  |
|                  | (0.00)     | (1.00)     | (1.00)     | (1.00)     |
| [Abu\textsuperscript{5,28}]CTX | 0.22±0.02  | 0.49±0.08  | 0.24±0.02  | 0.21±0.01  |
|                  | (0.97)     | (0.00)     | (0.84)     | (1.00)     |
| [Ser\textsuperscript{5,28}]CTX | 0.23±0.02  | 0.46±0.11  | 0.21±0.02  | 0.21±0.01  |
|                  | (0.89)     | (0.01)     | (0.98)     | (1.00)     |
| [Abu\textsuperscript{16,33}]CTX | 0.24±0.02  | 0.22±0.01  | 0.33±0.07  | 0.21±0.01  |
|                  | (0.67)     | (1.00)     | (0.13)     | (1.00)     |
| [Ser\textsuperscript{16,33}]CTX | 0.24±0.02  | 0.22±0.01  | 0.26±0.05  | 0.21±0.01  |
|                  | (0.77)     | (0.99)     | (0.70)     | (1.00)     |
| [Abu\textsuperscript{20,35}]CTX | 0.22±0.02  | 0.22±0.01  | 0.24±0.02  | 0.22±0.03  |
|                  | (0.99)     | (1.00)     | (0.80)     | (0.90)     |
| [Ser\textsuperscript{20,35}]CTX | 0.23±0.01  | 0.21±0.01  | 0.23±0.01  | 0.30±0.08  |
|                  | (0.99)     | (1.00)     | (1.00)     | (0.39)     |
| [Abu\textsuperscript{5,16,19,20,28,33,35}]CTX | 0.62±0.28  | 0.65±0.22  | 0.55±0.18  | 0.62±0.19  |
|                  | (0.04)     | (0.01)     | (0.01)     | (0.02)     |
| [Ser\textsuperscript{5,16,19,20,28,33,35}]CTX | 0.78±0.30  | 0.69±0.28  | 0.70±0.21  | 0.75±0.25  |
|                  | (0.01)     | (0.05)     | (0.00)     | (0.00)     |
| Levene’s P value | <.001      | <.001      | <.001      | <.001      |
| ANOVA F-Statistic| 14,501     | 9,781.4    | 16,558     | 4,418.3    |
| F (df1, df2)     | (10, 17,491) | (10, 17,445) | (10, 17,263) | (10, 17,498) |
| ANOVA p value    | <.001      | <.001      | <.001      | <.001      |

Abbreviations: Abu, L-α-aminobutyric acid; ANOVA, analysis of variance; CTX, chlorotoxin; df1, degrees of freedom between the groups; df2, degrees of freedom within the groups.

\textsuperscript{a} The mean±SD calculated CβHβ-to-CβHβ center-of-mass distance for the (Acetyl-Ala-Cys-Ala-NH\textsubscript{2})\textsubscript{2} dipeptide, as described in Appendix A, is 0.23±0.03 nm.

\textsuperscript{b} The CβHβ-to-CβHβ center-of-mass distance representing the 95% 1-sided CI for the (Acetyl-Ala-Cys-Ala-NH\textsubscript{2})\textsubscript{2} dipeptide, as described in Appendix A, is ≤0.26 nm.

The probabilities of finding water molecules within 0.5 nm of the terminal γ-sidechain atoms are shown in Table 3. The terminal Sγ of CTX remained relatively solvent shielded with a low probability of adjacent water molecules, regardless of its position within the native peptide. Substitution of a single Cys-Cys disulfide bond at the 2,19 and 5,28 positions tended to increase the probability of adjacent water molecules at the involved residues. For the hydrophobic Abu...
### Table 3. Probability ($\rho_{\gamma}(r)$) of finding water molecules within 0.5 nm of the terminal $\gamma$-sidechain atoms of Cys-, Abu-, and Ser- substituted residues ($S_\gamma$, $C_\gamma H_\gamma$, and $O_\gamma H_\gamma$, respectively)$^{a,b,c}$

| Peptide | Residue $\rho_{\gamma}(r)$ |
|---------|---------------------------|
|         | 2  | 5  | 16 | 19  | 20  | 28  | 33  | 35  |
| CTX     | 0.066 | 0.044 | 0.013 | 0.076 | 0.020 | 0.049 | 0.019 | 0.094 |
| [Abu$^{2,19}$]CTX | 0.173 | 0.016 | 0.012 | 0.113 | 0.022 | 0.037 | 0.017 | 0.086 |
| [Ser$^{2,19}$]CTX | 0.147 | 0.070 | 0.028 | 0.121 | 0.018 | 0.087 | 0.041 | 0.081 |
| [Abu$^{5,28}$]CTX | 0.077 | 0.144 | 0.013 | 0.084 | 0.015 | 0.060 | 0.012 | 0.081 |
| [Ser$^{5,28}$]CTX | 0.086 | 0.186 | 0.033 | 0.066 | 0.027 | 0.104 | 0.032 | 0.079 |
| [Abu$^{16,33}$]CTX | 0.090 | 0.035 | 0.078 | 0.059 | 0.025 | 0.044 | 0.053 | 0.070 |
| [Ser$^{16,33}$]CTX | 0.079 | 0.032 | 0.050 | 0.061 | 0.029 | 0.048 | 0.060 | 0.086 |
| [Abu$^{20,35}$]CTX | 0.075 | 0.032 | 0.031 | 0.093 | 0.038 | 0.052 | 0.024 | 0.111 |
| [Ser$^{20,35}$]CTX | 0.082 | 0.006 | 0.024 | 0.076 | 0.046 | 0.026 | 0.025 | 0.165 |
| [Abu$^{2,5,16,19,20,28,33,35}$]CTX | 0.101 | 0.138 | 0.101 | 0.138 | 0.126 | 0.124 | 0.123 | 0.138 |
| [Ser$^{2,5,16,19,20,28,33,35}$]CTX | 0.203 | 0.194 | 0.156 | 0.166 | 0.202 | 0.172 | 0.182 | 0.204 |

Abbreviations: Abu, L-$\alpha$-aminobutyric acid; CTX, chlorotoxin.

$^a$ The probability ($\rho_{\gamma}(r)$) of finding water molecules within 0.5 nm of the terminal $\gamma$-sidechain atom of Cys ($S_\gamma$) in the (Acetyl-Ala-Cys-Ala-NH$_2$)$_2$ dipeptide, as described in Appendix A Materials, is 0.114.

$^b$ The probability ($\rho_{\gamma}(r)$) of finding water molecules within 0.5 nm of the terminal $\gamma$-sidechain atoms of Abu ($C_\gamma H_\gamma$) in the Acetyl-Ala-Abu-Ala-NH$_2$ peptide, as described in Appendix A, is 0.190.

$^c$ The probability ($\rho_{\gamma}(r)$) of finding water molecules within 0.5 nm of the terminal $\gamma$-sidechain atoms of Ser ($O_\gamma H_\gamma$) in the Acetyl-Ala-Ser-Ala-NH$_2$ peptide, as described in Appendix A, is 0.213.
substitutions, this effect tended to be more local, with the exception of [Abu\textsuperscript{16,33}]CTX, which had an increase for Cys\textsuperscript{2}, and [Abu\textsuperscript{20,35}]CTX, with an increase for Cys\textsuperscript{2} and Cys\textsuperscript{16}. For the hydrophilic Ser substitutions, the effects appeared to be more widespread, with changes in hydration for other nonsubstituted Cys residues. The [Abu\textsuperscript{2,5,16,19,20,28,33,35}]CTX, and [Ser\textsuperscript{2,5,16,19,20,28,33,35}]CTX peptides showed significant increases in adjacent water molecules through the substituted residues.

2.5 Conformational Analysis

The lowest energy conformations were determined by projecting each trajectory onto the first 2 dihedral principal components, as show in Figure 5. The optimal number of clusters was selected by using a combination of visual inspection, sum of squared error, and silhouette plots, as shown in Figure B4. The lowest energy conformation of CTX was consistent with the \textsuperscript{1}H NMR structure: \(\alpha\)-helix from residues 15-20, 2 antiparallel \(\beta\)-sheets from residues 27-29 and 32-34, and 2 intervening turns from residues 22-25 and 30-31. There were 4 salt bridges: Arg\textsuperscript{14} and Asp\textsuperscript{15}, Lys\textsuperscript{15} and Asp\textsuperscript{19}, Arg\textsuperscript{25} and the C-terminus, and Lys\textsuperscript{27} and the C-terminus.

The lowest energy conformations of [Abu\textsuperscript{2}]CTX, [Ser\textsuperscript{2}]CTX, [Abu\textsuperscript{28}]CTX, and [Ser\textsuperscript{33}]CTX maintain secondary and tertiary structure that are consistent with the \(\alpha\)-\(\beta\)-motif and native CTX. This is despite the finding that the substituted residues’ sidechains remain separated and do not interact (Table 2). The [Ser\textsuperscript{16,33}]CTX peptide is unique in that the 2 Ser residues are in close proximity (Table 2) and most likely interact by hydrogen bonding to each other or through an associated water molecule, given the significant increase in \(\rho_{6(1)}\) (Table 3). The lowest energy conformations of [Ser\textsuperscript{5,28}]CTX, [Abu\textsuperscript{16,33}]CTX, [Abu\textsuperscript{20,35}]CTX, [Ser\textsuperscript{20,35}]CTX, [Abu\textsuperscript{2,5,16,19,20,28,33,35}]CTX, and [Ser\textsuperscript{2,5,16,19,20,28,33,35}]CTX deviate significantly from the \(\alpha\)-\(\beta\)-motif and native CTX, with changes in both their secondary and tertiary structure.

2.6 Essential Subspace Analysis

Figure 6 shows a comparison of sampled essential subspace and its normalized values. Despite the substantial changes that occurred in secondary and tertiary conformations with some of the selective and global Cys substitutions, particularly [Ser\textsuperscript{5,28}]CTX, [Abu\textsuperscript{16,33}]CTX, [Ser\textsuperscript{20,35}]CTX, [Abu\textsuperscript{2,5,16,19,20,28,33,35}]CTX, and [Ser\textsuperscript{2,5,16,19,20,28,33,35}]CTX, none of the nRMSIP values were 0.65 or less. This indicates that a significant degree of sampled conformational space is shared across all substituted peptides. The results can be divided into 4 classes for comparison: 0.65 \(\leq\) nRMSIP < 0.75, 0.75 \(\leq\) nRMSIP < 0.85, and 0.85 \(\leq\) nRMSIP < 0.95, and 0.95 \(\leq\) nRMSIP. Peptide pairwise comparisons sharing the greatest degree of subspace conformational sampling were CTX to [Ser\textsuperscript{2,19}]CTX, CTX to [Abu\textsuperscript{16,33}]CTX, CTX to [Abu\textsuperscript{20,35}]CTX, [Ser\textsuperscript{2,19}]CTX to [Abu\textsuperscript{2,19}]CTX, [Ser\textsuperscript{2,19}]CTX to [Abu\textsuperscript{20,35}]CTX, and [Abu\textsuperscript{16,33}]CTX to [Abu\textsuperscript{20,35}]CTX.

3. Discussion

Selective pairwise replacement of CTX Cys-Cys disulfide bonds: 2-19, 5-28, 16-33, and 20-35 with Abu, and complete 2, 5, 16, 19, 20, 28, 33, and 35 replacements with Abu, have been performed [29]. Native CTX with intact disulfide bonds had an early-eluting peak on analytic high-performance liquid chromatography (HPLC) consistent with greater exposure of hydrophilic and polar residues in the folded structure. The [Abu\textsuperscript{2,19}]CTX and [Abu\textsuperscript{5,28}]CTX analogs also had defined early-eluting peaks on HPLC but with lower intensity than native CTX, which indicated that removal of the disulfide bond at either Cys\textsuperscript{2}-Cys\textsuperscript{19} or Cys\textsuperscript{5}-Cys\textsuperscript{28} had some minor effects on the folded structure. The [Abu\textsuperscript{16,33}]CTX and [Abu\textsuperscript{20,35}]CTX analogs did not form distinct elution peaks on HPLC, which indicated the formation of non-native-like structures. Mass spectroscopy analysis of [Abu\textsuperscript{16,33}]CTX and [Abu\textsuperscript{20,35}]CTX also suggested multiple non-native-like disulfide bonds, which would indicate that a
Figure 5. The free energy landscape (kJ•mol⁻¹) as a function of the first 2 components of the dihedral principal component analysis (dPC1 and dPC2). The lowest energy conformations, as determined by cluster analysis, are shown and numbered in order from lowest to highest relative energy. The conformations have been rotated to optimally display the secondary structure elements and interactions.
Figure 6. Comparison of essential subspace sampling. Root-mean-square inner product RMSIP (top right in grayscale) and normalized RMSIP (bottom left in color) overlap matrix of trajectories of molecular dynamics simulation of all 11 chlorotoxin (CTX) peptides. By definition, the diagonal values equal 1.0.

The nativelike fold is not the most energetically favored conformation. CD measurements showed substantial structural similarity between CTX, [Abu$^{2,19}$]CTX, and [Abu$^{5,28}$]CTX suggestive of a folded $\alpha \beta$ motif. The CD spectrum of [Abu$^{2,5,16,19,20,28,33,35}$]CTX was more consistent with significant random coil conformation [29]. The authors did not report deconvolution of the CD spectra with the fraction of populated $\alpha$-helical, $\beta$-sheet, or bend/turn/coil conformations. Biological testing using a vascular endothelial growth factor–stimulated assay of cell migration indicated that CTX, [Abu$^{2,19}$]CTX, [Abu$^{5,28}$]CTX, and [Abu$^{2,5,16,19,20,28,33,35}$]CTX retain inhibitory activity, which suggested that the folded nativelike structure is not necessary for biological activity but does increase resistance to proteolytic degradation in vitro and in vivo [29].

The choice of Abu for substituting Cys residues has been driven by its isosteric and hydrophobic nature, with the goal of preserving the hydrophobic pocket in the peptide [27,85]. Because of the small and highly charged nature of these peptides, the surrounding environment of the Cys-Cys disulfide bond may not be as hydrophobic as previously considered. Examination of the rSASA plot of CTX in Figure B3 demonstrates that, although Cys residues are relatively solvent shielded, only the Cys$^2$ and Cys$^3$ residues are surrounded by either hydrophobic or aromatic residues. The remaining Cys residues are adjacent to charged residues such as Lys, Asp, or Arg, with the exception of Cys$^{33}$ which is adjacent to a hydrophilic Gln$^{32}$ and hydrophobic Leu$^{34}$. It is also
clear that Cys², Cys¹⁹, and Cys³⁵ are significantly less solvent shielded, whereas Cys¹⁶ and Cys³³ are the most solvent shielded as demonstrated by their rSASA values and their decreased probability of having water molecules within 0.5 nm (Table 3). In the current MD simulations, we were able to give detailed descriptions of the local hydrophobic/hydrophilic environment of each disulfide bond site.

Removal of the Cys²-Cys¹⁹ disulfide bond and replacement with either Abu or Ser has only minor effects on CTX secondary and tertiary conformation. Removal of this bond is well tolerated because the remaining Cys²-Cys⁵ bond maintains the N-terminus in close proximity with the antiparallel β-sheet. This interaction is also stabilized with an H-bond between the amine of Cys⁵ and the carbonyl of Pro⁵. Subtle differences are present between the Abu and Ser substitutions. Whereas Abu substitution allows for the 2 Abu sidechains to widely separate and the N-terminus to drift away slightly from the α-helix and β-sheet, Ser substitution allows for close approximation of the N-terminus to the distal strand of the β-sheet, forming a 3-strand antiparallel β-sheet with prominent H-bonds between the amine of Ser⁸ and the carbonyl of Pro⁰, the amine of Cys³³ and the carbonyl of Met³, the amine of Met³ and the carbonyl of Cys³³, and NεH of Gln³² and Oγ of Ser³. The sidechain of the Ser¹⁹ residue on the α-helix is positioned so that it can interact with the surrounding solvent. Substitution of the Cys²-Cys¹⁹ disulfide bond with Ser residues most likely removes some degree of tertiary strain within the peptide. Furthermore, it allowed more H-bonding interactions between the N-terminus and the antiparallel β-sheet. This is consistent with the disulfide bond between Cys²-Cys¹⁹ being more solvent exposed and thus favoring potential hydrophilic substitutions.

Substitution of the Cys⁵-Cys²⁸ disulfide bond allows the α-helix region to be lengthened from residues 9 through 20. The preserved Cys⁵-Cys¹⁹ bond, however, maintains the N-terminal region in close proximity with the distal α-helix and β-sheet. Due to elongation of the α-helix, the N-terminal region is not oriented in a way that would allow H-bonding to the distal strand of the β-sheet. Abu¹ and Abu² reside within a hydrophobic cleft between the α-helix and the proximal strand of the anti-parallel β-sheet interacting with the sidechain of Met². This preserves the hydrophobic region present in the native CTX but in a slightly less compact conformation. The conformational importance of this hydrophobic region is demonstrated by Ser substitution, which results in significant disruption of the α-helix with a large loop extending from Ser⁸ through Lys¹⁵ and a shift in the location of the antiparallel β-sheet from residues 25 through 29 and 32 through 36. This results in a conformation that exposes the involved residues to solvent and disrupts the native hydrophobic region.

Despite the high degree of solvent shielding at the disulfide bond between Cys¹⁶-Cys³³, substitution with the hydrophobic Abu was not tolerated and resulted in nonnative isomers in oxidative folding experiments [28,29]. There is significant disruption of the secondary and tertiary conformations of the [Abu¹⁶,³³]CTX peptide. As noted above, the α-helix is disrupted and the N-terminus is dominated by antiparallel β-sheet formation in the lowest energy conformations. The lack of a disulfide bond between Cys¹⁶-Cys³³ disrupts the association between the α-helical region and the terminal strand of the antiparallel β-sheet. This is particularly important because the sidechains of the antiparallel β-sheet region are oriented away from the α-helix and toward the solvent, and with the exception of Abu¹⁶ and Cys³³, none of the α-helix residues are aligned to interact with the back surface of the β-sheet. As shown in Figure B2, the lack of H-bond donor and acceptor groups between the α-helix and β-sheet regions also makes the association between the α-helix and β-sheet strongly dependent on the presence of the disulfide bond between residues Cys¹⁶ and Cys³³. This substitution also results in closer proximity of the Cys²-Cys¹⁹, Cys⁵-Cys²⁸, and Cys³⁵ disulfide bonds, which may explain why multiple isomers occur with synthesis [29]. Ser substitution of these residues, however, preserves the overall secondary and tertiary conformation. The elongation of the α-helix appears to stabilize interaction between the two Ser residues and an associated water molecule.

Substitution at the Cys²⁰ and Cys³⁵ residues appears to allow preservation of the overall secondary and tertiary conformation with some degree of elongating of the α-helical region. Although the overall fold of the peptide is preserved, multiple H-bonds occur between the α-helix
and β-sheets that do not occur in the native peptide, and the resulting fold places the Cys²-Cys¹⁹, Cys²-Cys²⁵, and Cys¹⁶-Cys³⁰ residues in close proximity and, like the Cys¹⁶-Cys³ substitution, may explain why multiple isomers occur with synthesis. The result for Ser substitution is similar, except that the C-terminal antiparallel β-sheet is disrupted.

4. Materials and Methods

4.1 Initial Peptide Structures

All MD simulations were performed with the GROMACS 5.1.2 software packages using the CHARMM36m force field parameters with the CHARMM36m consistent version of TIP3P water [33-44]. The Leonard-Jones and electrostatic potentials for the atoms of the Abu residue were assigned in a manner consistent with other noncyclic aliphatic residues (Ala, Leu, Ile, and Val) within the CHARMM36m force field and are provided in Appendix A [33-36]. The initial structure of CTX for the simulation was the first conformation of the published ¹H NMR solution structure (Protein Data Bank ID: 1CHL) [30]. Starting structures of the peptides [Abu²⁻¹⁹]CTX, [Ser²⁻¹⁹]CTX, [Abu²⁻¹⁸]CTX, [Ser²⁻¹⁸]CTX, [Abu¹⁶⁻¹³]CTX, [Ser¹⁶⁻¹³]CTX, [Abu²⁰⁻³⁷]CTX, [Ser²⁰⁻³⁷]CTX, and [Ser²⁻¹⁶]CTX were obtained by changing the respective residues from Cys to Abu or Ser using YASARA [45]. The protonation state and charges of all residues within the peptides were set to correspond to a pH of 7.0.

4.2 Molecular Dynamics

Peptides were solvated in dodecahedral boxes with TIP3P water with 150 mM NaCl. Additional Cl⁻ and Na⁺ ions were used to neutralize the charges of the systems. The minimum distance of the peptide to the edge of the dodecahedron was 1.4 nm, with the exception of [Abu²⁻¹⁵]CTX and [Ser²⁻¹⁵]CTX, which required a 2.0 nm minimum distance of the peptide to the edge of the dodecahedron to prevent interaction of the peptide with its periodic image. The solvated systems were subjected to 5,000 steps of steepest descent energy minimization without restraints, allowing all bond distances and angles to relax. NVT (constant number, volume, and temperature) simulations of the positionally restrained peptides (force constant, 1,000 kJ·mol⁻¹) were performed for 10 nanoseconds at 310 K, followed by constant number, pressure and temperature (NPT) simulations of the positionally restrained peptides for 10 nanoseconds at 310 K and 101.325 kPa. The temperature and pressure, respectively, were kept constant by the stochastic velocity-rescaling method of Bussi et al. and the method of Berendsen et al. [46,47]. The relaxation constant was 0.1 picosecond and the isothermal compressibility was 4.5×10⁻⁵ bar⁻¹. We used 2 femtoseconds for the integration step and the LINCS algorithm for constraining all bonds to their correct length; the warning angle was 30° [48,49]. The particle mesh Ewald method was used to calculate long-range electrostatic interactions; cutoff distances were 1.2 nm and 1.0 nm, and the Fourier spacing was 0.15 nm [50]. The switch method was used to calculate van der Waals interactions, the short-range and long-range cutoffs were 1.0 and 1.2 nm, respectively.

Production runs of 4.2-microsecond NPT simulations were performed at 310 K and 101.325 kPa. The peptides and solvent with ions were separately coupled to a Parrinello-Rahman barostat and the temperature of the peptides and solvent separately maintained by the stochastic velocity-rescaling method [46,51]. The integration step, bond and angle constraints, long-range electrostatic interactions, and van der Waals interactions were calculated as described above.

4.3 Trajectory Analysis

The first 200 nanoseconds of each peptide simulation was considered system equilibration, and the subsequent 4 microseconds was used for analysis with a sampling frequency of 0.1 nanosecond.

4.3.1 Biophysical Properties
The radius of gyration ($R_g$), Ca-α-trace RMSD from the 1H NMR solution conformation of CTX (RMSD$_{CTX}$), Ca-α-trace RMSD from the average sampled peptide conformation (RMSD$_{AVG}$), per-residue Ca-α-trace root-mean-square fluctuation (RMSF) from the average sampled peptide conformation, global and per-residue fraction of sampled secondary structure (α-helix, β-sheet, and bend/turn), and solvent-accessible surface area (SASA), hydrophobic SASA (SASA$_{hydrophilic}$), and hydrophobic SASA (SASA$_{hydrophilic}$) components were calculated using g$\_gyration$, g$\_rmsd$, g$\_rmsf$, do$\_dssp$, and g$\_sasa$ utilities of GROMACS, respectively [31,43]. The SASA of the peptides was calculated using the atomic radii of Lee and Richards with a probe radius of 0.14 nm and 1,000 points-per-sphere resolution [52].

4.3.2 Interactions

The per-residue SASA was normalized to values of the maximal SASA as determined from MD simulations on Acetyl-Ala-Xaa-Ala-NH$_2$ (where Xaa is either Ser or Abu) and (Acetyl-Ala-Cys-Ala-NH$_2$)$_2$ (dimer with Cys-Cys disulfide bond) [53,54]. Since the published data sets do not contain corresponding values for Abu, control simulations and calculations were performed as outlined in Appendix A (Table A1 and Figures A1 through A5). The change in per-residue SASA ($\Delta$SASA) between the native CTX and Abu- or Ser-substituted peptides was calculated as:

$$\Delta\text{SASA} = \text{SASA}_{\text{Substituted}} - \text{SASA}_{\text{CTX}}$$

The intrachain sidechain-sidechain (SC-SC) and backbone-backbone (BB-BB) contacts and H-bonds were calculated with the g$\_mdmat$ and g$\_hbond$ utilities of GROMACS [43]. SC-SC and BB-BB contacts were designated for interatomic distances (between the heavy atoms of a residue) of 0.5 nm or less; H-bonds were designated for a donor-acceptor radius of 0.35 nm or smaller and a donor-hydrogen-acceptor angle of 30° or less. 1H NMR cannot measure the sulfur-to-sulfur interatomic distance, and assignment of the spectra and calculation of the peptide's conformations is dependent on the presence of a Cys-Hβ to bonded Cys-Hβ nuclear Overhauser effect spectral peak; therefore, interactions between bound Cys and Abu- or Ser-substituted residues were determined by measuring the CβHβ to CβHβ center-of-mass distance and calculating the probability of contact within 0.5 nm [30,55]. Hydration of the sidechains of Cys, Abu, and Ser was determined by integrating the radial distribution function for water molecules within 0.5 nm of the sidechain. The integral of this function is equal to the probability of finding water molecules within the defined radius [56,57].

4.3.2 Conformational Analysis

The sampled conformations of the peptides were analyzed using the dihedral principal component analysis method [58,59]. The time-dependent φ/ψ dihedral angles from residues 2 to 35 of the peptides were extracted from the trajectories using the g$\_rama$ utility of GROMACS, and an in-house python script was used to transform the data for the input to the dihedral principal component analysis program (provided by Dr. Yuguang Mu). We identified the lowest energy conformations by projecting the trajectories of the first 2 principal components onto a 2-dimensional free energy ($\Delta$G) landscape:

$$\Delta G = -R \cdot T \cdot \ln \frac{\rho_{xy}}{\rho_{\text{max}}}$$

where R is the universal gas constant, T is the temperature, and x and y are the first 2 dihedral principal components from the trajectory. The ΔG landscape was calculated by dividing the principal component 1 – principal component 2 subspace into grids to create a 2-dimensional histogram of the sampled phase space and calculating the probability $\rho_{x,y}$ using an in-house python
script, with \( \rho_{\text{max}(x,y)} \) corresponding to the grid with the maximum probability of occurrence. Results were visualized using the scatterplot3D, akima, and latticeExtra packages in the R software environment, with conformations and secondary structural elements rendered using YASARA [45,60-63]. Families of low-energy conformations were identified using k-means clustering as implemented in the cluster package in R, and the identified lowest energy conformations were extracted for further analysis [63,64]. The optimal number of clusters was determined by visual inspection, sum of squared error, average silhouette width, silhouette coefficient, and distribution plots, as shown in Figure B4 [65-68].

4.3.4 Essential Subspace Analysis

The overlap of the sampled essential subspace of trajectories for different peptides as a function of Cys-Cys substitution was determined by calculating the root-mean-square inner product (RMSIP):\[
\text{RMSIP}_{AB} = \left( \frac{1}{N} \sum_{i=1}^{N} \sum_{j=1}^{N} (\eta_i^A \cdot \eta_j^B)^2 \right)^{\frac{1}{2}}
\]

comparing MD trajectories of peptides A and B, where \( \eta_i^A \) and \( \eta_j^B \) are the respective eigenvectors of the sampled essential subspace, and N is the total number of eigenvectors to be considered [69-72]. To correct for sampling errors and autocorrelation, the RMSIP can be normalized (nRMSIP):

\[
n\text{RMSIP} = \frac{\text{RMSIP}_{A \cdot B}}{\sqrt{\text{RMSIP}_{A1 \cdot A2} \cdot \text{RMSIP}_{B1 \cdot B2}}} \tag{4}
\]

RMSIP\(_{A1 \cdot A2}\) and RMSIP\(_{B1 \cdot B2}\) are comparisons between the first and second halves of MD trajectories for peptides A and B, respectively. The nRMSIP ranges from 0 to 1; nRMSIP values approaching 1 indicate that the sampled essential subspaces of peptides A and B are similar, whereas lower values indicate differences between the sampled essential subspace of peptides A and B that cannot be explained by sampling alone. An nRMSIP of 0 indicates that the sampled essential subspaces of peptides A and B are orthogonal [72].

4.4 Statistical Analysis

The biophysical properties \( R_g \), RMSD\(_{\text{CTX}}\), RMSD\(_{\text{AVG}}\), fractions of populated secondary structure (\( \alpha \)-helix, \( \beta \)-sheet, and \( \beta \)-bend/turn and coil), SASA, SASA\(_{\text{Hydrophobic}}\), SASA\(_{\text{Hydrophilic}}\), and Cys and substituted Cys C\( \beta \)-H\( \beta \)-to-C\( \beta \)-H\( \beta \) center-of-mass distances were characterized for each peptide and reported as mean ± standard deviation. The homoscedasticity (homogeneities of variances) within the data were determined with Levene’s test and comparisons made with analysis of variance (ANOVA) or Welch’s ANOVA where appropriate (Levene’s \( P < .05 \)).

There were a total of 55 pairwise comparisons (N):

\[
N = \frac{n(n - 1)}{2} \tag{5}
\]

where \( n \) is the number of peptides in the series. The ANOVA and Welch’s ANOVA are statistically robust methods of identifying differences between the means of the sampled populations, but they cannot determine which pairwise comparison of the means is statistically significant. Therefore, we
used a post hoc analysis with the Games-Howell test as implemented in the car package in R [63, 73-84].

5. Conclusions

In CTX, the disulfide bonds between Cys₁⁶-Cys³₃ and Cys²₀-Cys³₅ are required to maintain the association between the α-helical region from residues 13 through 20 and the 2 antiparallel β-sheets from residues 27 through 29 and 32 through 34. This conformational dependence arises from the lack of SC-SC and BB-BB interactions between the 3 components of the motif, either from hydrophobic, aromatic, salt bridge, or H-bond formation. Of these 2 disulfide bonds, the bond between Cys₁⁶ and Cys³₃ appears to be more critical, allowing for wider separation of the α-helix and β-sheet, whereas replacement of the Cys²₀ and Cys³₅ residues causes less conformational disruption. Selective use of Ser₁⁶,₃₃ may mitigate this destabilizing effect, since an interaction may occur between the 2 residues and an associated water molecule. Although stepwise removal of disulfide bonds or all disulfide bonds increases protease degradation, it has a less marked effect on biological activity, as demonstrated by Ojeda et al. [29]. The binding region of CTX to its purported receptor may be independent of a restricted conformation. It should also be noted that even with removal of all 4 Cys-Cys disulfide bonds, the peptides still share a substantial degree of conformational space sampling, and short, appropriately ordered segments of the peptide may present successfully to the receptor to initiate binding.

Supplementary Materials: Supplementary materials can be found at www.mdpi.com/xxx/s1.

Author Contributions: conceptualization, C.R.W and S.L.; methodology, C.R.W and S.L.; software, C.R.W., A.J.G., L.V.-O., and S.L.; validation, C.R.W., A.J.G. and L.V.-O.; formal analysis, A.J.G. and L.V.-O.; investigation, A.J.G. and L.V.-O.; resources, C.R.W.; data curation, A.J.G. and L.V.-O.; writing—original draft preparation, C.R.W.; writing—review and editing, C.R.W., A.J.G. and L.V.-O.; visualization, A.J.G. and L.V.-O.; supervision, C.R.W.; project administration, C.R.W.; funding acquisition, C.R.W.

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Conflicts of Interest: Dr. Charles R. Watts is a consultant for Medtronic Spine and Biologics. The remaining authors have disclosed that they do not have any conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Abbreviations

Abu L-α-aminobutyric acid
ANOVA analysis of variance
BB-BB backbone-backbone
CTX chlorotoxin
CD circular dichroism
HPLC high-performance liquid chromatography
MD molecular dynamics
nRMSIP normalized root-mean-square inner product
NMR  nuclear magnetic resonance spectroscopy
NPT  constant number, pressure, and temperature
R_g  radius of gyration
rSASA  relative solvent-accessible surface area
RMSD  root-mean-square deviation
RMSF  root-mean-square fluctuation
RMSIP  root-mean-square inner product
SASA  solvent-accessible surface area
SC-SC  sidechain-sidechain
SD  standard deviation

Appendix A

A.1 Assignments of Force Field Parameters for L-α-Aminobutyric Acid (Abu)

The Leonard-Jones and electrostatic potentials for the atoms of the Abu residue were assigned in a manner consistent with other noncyclic aliphatic residues within the CHARMM36m force field (Ala, Leu, Ile, and Val) [33-36]. The following lines were added to the merged.rtp file in the charmm36-june2015.ff directory tree of GROMACS. Standard CMAP potentials were used for the \( \phi, \psi \) dihedral angles and no modifications were made to the cmap.itp file within the charmm36-june2015.ff directory.

```plaintext
[ ABU ]
[ atoms ]
N    NH1  -0.470 0
HN   H    0.310 1
CA   CT1  0.070 2
HA   HB1  0.090 3
CB   CT2  -0.180 4
HB1  HA2  0.090 5
HB2  HA2  0.090 6
CG   CT3  -0.270 7
HG1  HA3  0.090 8
HG2  HA3  0.090 9
HG3  HA3  0.090 10
C    C    0.510 11
O    O    -0.510 12
[ bonds ]
CB   CA
CG   CB
N    HN
N    CA
C    CA
C    +N
CA   HA
CB   HB1
CB   HB2
```
A.2 Biophysical Properties of L-α-Aminobutyric Acid (Abu)

The solvent-accessible surface area (SASA) and radial distribution functions (Table A1) of the terminal methyl (Abu), hydroxyl (Ser), and disulfide (Cys-Cys) unfolded residues were determined in the following manner. The Acetyl-Ala-Xaa-Ala-NH₂ (where Xaa is either Ser or Abu) and (Acetyl-Ala-Cys-Ala-NH₂)₂ (bridged with a Cys-Cys disulfide bond) peptides were simulated using 0.5 µs NPT molecular dynamics runs at 310 K and 101.325 kPa. Fully extended initial conformations were created using the YASARA program [47]. The peptides were separately solvated in dodecahedral boxes with TIP3P water and 150 mM NaCl. The solvated conformations were energy-minimized by 5000 steps of steepest descent without restraint allowing all bond distances and angles to relax. NVT simulations of the positional restrained peptides (force constant of 1000 kJ•mol⁻¹) were performed for 10 ns at 310 K followed by NPT simulations of the positional restrained peptides for 10 ns at 310 K and 101.325 kPa stochastic velocity-rescaling method of Bussi for the temperature and the method of Berendsen for the pressure with separate temperature and pressure baths for the peptide and solvent and relaxation constants of 0.1 ps and 4.5 X 10⁻⁵ bar⁻¹ isothermal compressibility [48,49]. The production runs of 500 ns NPT simulations were performed at 310 K and 101.325 kPa pressure. The peptide and solvent with ions were separately coupled to trans and cis Parrinello-Rahman barostats and the temperatures were maintained by separate coupling to stochastic thermostats using the velocity-rescaling method of Bussi-Parrinello [48,53]. The long-range electrostatic interactions were calculated using the PME method with a 1.2 nm cutoff distance and 0.15 nm Fourier spacing [52]. For the calculations of van der Waals interactions the switch method was used and the short-range and long-range cutoff, respectively, was 1.0 and 1.2 nm. The integration step was 2 fs; the LINCS algorithm was used to constrain all bonds to their correct length, with a warning angle of 30° [48,49].

Table A1. Residue solvent exposed surface area (SASA) Abu, Ser, and Cys-Cys; and probability ($p_{g(r)}$) of finding water molecules within 0.5 nm distance of the terminal methyl (Abu), hydroxyl (Ser), and disulfide (Cys) moieties. For the Cys-Cys disulfide bond the monomer value is given first and the dimer in parenthesis.

| Residue | SASA /nm² | $p_{g(r)}$ |
|---------|-----------|------------|
| Abu     | 1.347 ± 0.121 | 0.190 |
| Ser     | 1.219 ± 0.111 | 0.213 |
| Cys-Cys | 0.758 ± 0.142 (1.515 ± 0.283) | 0.114 |

Standard quality assurance of the trajectories was performed and calculations of biophysical properties as outlined in the methods section of the manuscript Figures A1 through Figure A3. The first 100 ns of each peptide simulation was considered system equilibration and the subsequent 500 ns used for analysis with a sampling frequency of 0.1 ns.
Figure A1. Cα-trace configurational entropy for Acetyl-Ala-Abu-Ala-NH₂ (A), Acetyl-Ala-Ser-Ala-NH₂ (B), and (Acetyl-Ala-Cys-Ala-NH₂)₂ (C) as a function of time.
Figure A2. The sampled φ-ψ dihedral angles of Abu (A), Ser (B), and Cys-Cys disulfide bond (C). The color scale bars represent the global probability of a given φ-ψ dihedral angle.
Figure A3. Radius of gyration ($R_g$) for Acetyl-Ala-Abu-Ala-NH$_2$ (A), Acetyl-Ala-Ser-Ala-NH$_2$ (B), and (Acetyl-Ala-Cys-Ala-NH$_2$)$_2$ (C) as a function of time.

The time-averaged SASA and radial distribution plots representing the probability of finding water molecules within 0.5 nm of the terminal moiety of Cys, Ser, and Abu are displayed graphically in Figures A4 and A5.
Figure A4. Solvent Accessible Surface Area (SASA) for Abu (A), Ser (B), and Cys-Cys disulfide bond (C) as a function of time.
Figure A5. Radial distribution function of water from the terminal methyl, hydroxyl and disulfide moieties of Acetyl-Ala-Abu-Ala-NH$_2$ (A), Acetyl-Ala-Ser-Ala-NH$_2$ (B), and (Acetyl-Ala-Cys-Ala-NH$_2$) (C) respectively. The probability of finding water molecules surrounding the respective moieties was determined by integrating the radial distribution function within 0.5 nm distance.
Appendix B

Table B1 Non-statistically significant comparisons between calculated biophysical properties and the peptides as determined by Games-Howell post-hoc analysis, all other comparisons were considered statistically significant (p < 0.05).

| Property                  | Compared Peptides                  | Mean Difference | 95% CI          | p-value |
|---------------------------|-----------------------------------|-----------------|-----------------|---------|
| $R_g$/nm                  | [Abu$^{2,19}$]CTX vs [Abu$^{16,33}$]CTX | 0.0012          | -0.0015 to 0.0039 | 0.94    |
|                           | [Abu$^{5,28}$]CTX vs [Ser$^{2,19}$]CTX | 0.0009          | -0.0013 to 0.0030 | 0.97    |
| RMSD$_{CTX}$/nm           | [Abu$^{2,20}$]CTX vs [Abu$^{16,33}$]CTX | 0.0002          | -0.0014 to 0.0018 | 1.00    |
|                           | [Abu$^{5,33}$]CTX vs [Ser$^{2,19}$]CTX | 0.0004          | -0.0010 to 0.0018 | 1.00    |
|                           | [Abu$^{5,33}$]CTX vs [Ser$^{5,28}$]CTX | 0.0014          | -0.0001 to 0.0028 | 0.10    |
|                           | [Abu$^{5,33}$]CTX vs [Ser$^{2,5,16,19,20,28,33,35}$]CTX | 0.0006         | -0.0009 to 0.0019 | 0.97    |
|                           | [Ser$^{2,19}$]CTX vs [Ser$^{5,28}$]CTX | -0.0010         | -0.0004 to 0.0023 | 0.43    |
|                           | [Ser$^{5,28}$]CTX vs [Ser$^{5,28}$]CTX | 0.0004          | -0.0018 to 0.0010 | 1.00    |
|                           | [Ser$^{5,28}$]CTX vs [Ser$^{2,5,16,19,20,28,33,35}$]CTX | -0.0002        | -0.0014 to 0.0011 | 1.00    |
|                           | [Ser$^{5,28}$]CTX vs [Ser$^{5,28}$]CTX | 0.0008          | -0.0005 to 0.0022 | 0.69    |
|                           | [Ser$^{16,33}$]CTX vs [Ser$^{5,28}$]CTX | 0.0012          | -0.0001 to 0.0025 | 0.09    |
| RMSD$_{Avg}$/nm           | [Abu$^{2,19}$]CTX vs [Ser$^{2,5,28}$]CTX | 0.0013          | -0.0001 to 0.0027 | 0.07    |
| $\alpha$-helix /$\rho$ CTX | [Abu$^{2,19}$]CTX vs [Ser$^{5,28}$]CTX | 0.0047          | -0.0001 to 0.0095 | 0.07    |
|                           | [Abu$^{5,28}$]CTX vs [Ser$^{2,5,16,19,20,28,33,35}$]CTX | -0.0047        | -0.0106 to 0.0012 | 0.27    |
|                           | [Ser$^{2,19}$]CTX vs [Ser$^{5,28}$]CTX | -0.0045         | -0.0099 to 0.0001 | 0.24    |
| $\beta$-sheet /$\rho$     | [Abu$^{2,20}$]CTX vs [Abu$^{20,35}$]CTX | -0.0022         | -0.0047 to 0.0002 | 0.12    |
|                           | [Abu$^{5,33}$]CTX vs [Ser$^{2,28}$]CTX | -0.0005         | -0.0048 to 0.0038 | 1.00    |
|                           | [Abu$^{5,28}$]CTX vs [Ser$^{5,28}$]CTX | 0.0017          | -0.0024 to 0.0058 | 0.97    |
| Bend/turn /$\rho$         | [Abu$^{2,5,16,19,20,28,33,35}$]CTX vs [Ser$^{16,33}$]CTX | 0.0039         | -0.0041 to 0.0120 | 0.90    |
| SASA /nm$^2$              | [Abu$^{2,19}$]CTX vs [Abu$^{16,33}$]CTX | 0.066           | -0.041 to 0.172  | 0.66    |
|                           | [Abu$^{2,20}$]CTX vs [Ser$^{16,33}$]CTX | -0.061          | -0.175 to 0.053  | 0.82    |
| SASA$_{hydrophobic}$/nm$^2$ | [Abu$^{2,5,16,19,20,28,33,35}$]CTX vs [Ser$^{2,19}$]CTX | -0.049          | -0.14 to 0.039   | 0.78    |
| SASA$_{hydrophilic}$/nm$^2$ | [Abu$^{2,5,16,19,20,28,33,35}$]CTX vs [Ser$^{5,28}$]CTX | -0.004         | -0.089 to 0.081  | 1.00    |
|                           | [Ser$^{16,33}$]CTX vs [Ser$^{20,35}$]CTX | -0.002          | -0.079 to 0.076  | 1.00    |
Figure B1. The per-residue Ca-trace RMSF (black) overlaid with the average conformation of the peptide itself (black) and with the average conformation of CTX (red).
Figure B2. The fraction of populated H-bonds normalized to the total H-bonds observed during the simulation is plotted by residue.
Figure B3. The relative solvent accessible surface area ($\Delta r\text{SASA}$ in black) and change in relative solvent accessible surface area ($\Delta \Delta r\text{SASA}$ in red) compared to CTX as a function of residue number.
Table B2. Non-statistically significant comparisons between calculated center of mass distances between CβHβ and CβHβ atoms for the peptides as determined by Games-Howell post-hoc analysis, all other comparisons were considered statistically significant (p < 0.05).

| Cystine Bond | Compared Peptides       | Mean Difference | 95% CI          | p-value |
|--------------|-------------------------|-----------------|-----------------|--------|
| 2-19         | CTX [Abu^{16,33}]CTX   | -0.0006         | -0.0020 to 0.0008 | 0.96   |
| [Abu^{5,28}]CTX | [Abu^{20,35}]CTX     | 0.0003          | -0.0001 to 0.0015 | 1.00   |
| 5-28         | CTX [Ser^{20,35}]CTX  | -0.0005         | -0.0011 to 0.0003 | 0.62   |
| [Abu^{2,19}]CTX | [Ser^{2,19}]CTX       | -0.0009         | -0.0021 to 0.0002 | 0.27   |
| [Abu^{2,19}]CTX | [Ser^{16,33}]CTX      | 0.0004          | -0.0008 to 0.0015 | 1.00   |
| 16-33        | CTX [Ser^{2,19}]CTX   | 0.0004          | -0.0023 to 0.0031 | 1.00   |
| [Ser^{16,33}]CTX | [Ser^{20,35}]CTX     | 0.0001          | -0.0008 to 0.0006 | 1.00   |
| 20-35        | CTX [Abu^{2,19}]CTX   | 0.0004          | -0.0009 to 0.0002 | 0.58   |
| [Abu^{16,33}]CTX | [Ser^{2,19}]CTX       | -0.0005         | -0.0011 to 0.0001 | 0.31   |
| [Abu^{16,33}]CTX | [Ser^{16,33}]CTX   | 0.0002          | -0.0004 to 0.0008 | 0.98   |
Figure B4. Sum of Squared Error (SSE), Average Silhouette width (\(S_{AVG}\)), Silhouette Coefficient (SC) and cluster distribution plots corresponding to the dihedral principle component conformational analysis presented in Figure 7 of the manuscript for: CTX (A), [Abu^{2,19}]CTX (B), [Ser^{2,19}]CTX (C), [Abu^{5,28}]CTX (D), [Ser^{5,28}]CTX (E), [Abu^{16,33}]CTX (F), [Ser^{16,33}]CTX (G), [Abu^{20,35}]CTX (H), [Ser^{20,35}]CTX (I), [Abu^{2,5,16,19,20,28,33,35}]CTX (J), and [Ser^{2,5,16,19,20,28,33,35}]CTX (K). The selected cluster number is shown in red with its corresponding average silhouette coefficient.
Figure B4. Sum of Squared Error (SSE), Average Silhouette width ($S_{AVG}$), Silhouette Coefficient (SC) and cluster distribution plots corresponding to the dihedral principle component conformational analysis presented in Figure 7 of the manuscript for: CTX (A), [Abu$^{2,19}$]CTX (B), [Ser$^{2,19}$]CTX (C), [Abu$^{5,28}$]CTX (D), [Ser$^{5,28}$]CTX (E), [Abu$^{16,33}$]CTX (F), [Ser$^{16,33}$]CTX (G), [Abu$^{20,35}$]CTX (H), [Ser$^{20,35}$]CTX (I), [Abu$^{2,5,16,19,20,28,33,35}$]CTX (J), and [Ser$^{2,5,16,19,20,28,33,35}$]CTX (K). The selected cluster number is shown in red with its corresponding average silhouette coefficient.
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