Protein Structure and Folding: Structural Insights into the Role of the Cyclic Backbone in a Squash Trypsin Inhibitor

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Structural Insights into the Role of the Cyclic Backbone in a Squash Trypsin Inhibitor*

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Background: MCoTI-II is a potent serine protease inhibitor with a cyclic protein backbone.

Results: Its three-dimensional structure and dynamics in complex with trypsin were determined using x-ray and NMR methods.

Conclusion: The cyclic backbone of MCoTI-II participates in favorable interactions with trypsin.

Significance: We demonstrate a new role for backbone cyclization in enhancing enzyme inhibitory activity.

MCoTI-II is a head-to-tail cyclic peptide with potent trypsin inhibitory activity and, on the basis of its exceptional proteolytic stability, is a valuable template for the design of novel drug leads. Insights into inhibitor dynamics and interactions with biological targets are critical for drug design studies, particularly for protease targets. Here, we show that the cyclization and active site loops of MCoTI-II are flexible in solution, but when bound to trypsin, the active site loop converges to a single well-defined conformation. This finding of reduced flexibility on binding is in contrast to a recent study on the homologous peptide MCoTI-I, which suggested that regions of the peptide are more flexible upon binding to trypsin. We provide a possible explanation for this discrepancy based on degradation of the complex over time. Our study also unexpectedly shows that the cyclization loop, not present in acyclic homologues, facilitates potent trypsin inhibitory activity by engaging in direct binding interactions with trypsin.

Both MCoTI-I and II are potent trypsin inhibitors isolated from the seeds of Momordica cochinchinensis and are members of the squash trypsin inhibitor family (1, 2). Peptides in this family contain ~30 amino acids and have a cystine knot (or knottin) arrangement of their three conserved disulfide bonds. In contrast to the majority of squash trypsin inhibitors, MCoTI-I and II both contain an amide-cyclized backbone and consequently are classified as cyclotides (3), ultra-stable plant defense miniproteins that contain a cystine knot and cyclic backbone. This unique structural motif, present only in the cyclotides, is referred to as a cyclic cystine knot (4).

Cyclotides are gene-encoded, and although the organization of the genes differs in different plants, the common feature is a conserved Asn/Asp residue at the C-terminal of the mature peptide sequence (5–8). This C-terminal residue appears to be critical for cyclization via an asparaginyl endopeptidase. The sequences of selected cyclotides, including MCoTI-I and II, are shown in Fig. 1A. There are six backbone loops between the cysteine residues, with loop 6 forming as a result of the cyclization. MCoTI-I and II were the first cyclic peptides isolated from M. cochinchinensis, but three additional cyclic peptides with homologous sequences to MCoTI-I and II have recently been discovered in this plant (7). A database (Cybase) (9) has been established to collate the sequences of cyclotides, and there are now >250 sequences characterized.

Both MCoTI-I and MCoTI-II are stable in human serum, with MCoTI-I reported to have a half-life of more than 2 days (10, 11). The stability of cyclotides, coupled with their high sequence diversity, has led to the suggestion they might be useful in the design of novel drug leads (12). In support of this suggestion, there are now several examples of the use of cyclotides as templates, including re-engineering kalata B1, the prototypical cyclotide, to incorporate and stabilize a bioactive peptide sequence with anti-cancer activity (13). Similarly, recent studies have shown that MCoTI-II can be used as a template to develop therapeutic angiogenic agents (11), as well as inhibitors of β-tryptase and human leukocyte elastase, therapeutic targets in inflammatory disorders (14, 15). MCoTI-I has been used to design inhibitors of HIV-1 viral replication (16) and a compound that activates the p53 tumor suppressor pathway in vivo (10). The latter example is an intracellular target, consistent with the finding that MCoTI-II is able to penetrate cells (17).

To exploit the potential of cyclotide frameworks such as MCoTI-II in drug delivery, it is important to understand their structure and dynamics. Several three-dimensional structures...
of cyclotides have been determined using NMR spectroscopy, and they generally highlight the well-defined structures present in this family (18). Surprisingly, however, the solution structure of MCoTI-II showed loop 6 to be highly disordered as shown in Fig. 1B (3, 19). Although the structures of a range of cyclotides have been determined, there have been limited studies on the dynamics and interactions of cyclotides with biological targets.

The most extensive analysis was done on MCoTI-I (20) where analysis of the dynamics with NMR spectroscopy suggested a lower order parameter for the cyclization loop (loop 6), indicative of greater structural flexibility for this region of the peptide compared with other loops. Interestingly, an analysis of the dynamics of MCoTI-I bound to trypsin was also carried out, and it was reported that several residues, including the active site lysine residue, become more flexible upon binding to trypsin (20). This is a very surprising result given that the MCoTI-II sequence and structure are similar to those of MCoTI-I. The most extensive analysis was done on MCoTI-I (20) where analysis of the dynamics with NMR spectroscopy suggested a lower order parameter for the cyclization loop (loop 6), indicative of greater structural flexibility for this region of the peptide compared with other loops. Interestingly, an analysis of the dynamics of MCoTI-I bound to trypsin was also carried out, and it was reported that several residues, including the active site lysine residue, become more flexible upon binding to trypsin (20). This is a very surprising result given that the MCoTI-II sequence and structure are similar to those of MCoTI-I.

In this study, we have analyzed the dynamics of MCoTI-II in solution and determined its crystal structure bound to trypsin. This is the first structure of a peptide containing a cyclic cystine knot motif bound to trypsin and, indeed, the first resolution of the structure of any cyclotide bound to a defined macromolecular target. Although MCoTI-II differs from MCoTI-I by just two residues (Fig. 1A), our results differ strikingly with the study by Puttamadappa et al. (20) and definitively reveal a tight rigid complex formed between trypsin and MCoTI-II. We believe that the earlier result of an apparently looser structure on binding probably reflects a previously undetected cleavage reaction in the complex. Furthermore, our study reveals a number of interactions between the cyclization loop and trypsin and provides new insights into the role of the cyclic backbone in enhancing the potency of protease inhibitors.

**EXPERIMENTAL PROCEDURES**

**Extraction of MCoTI-II from M. cochinchinensis**—Native MCoTI-II was purified from the seeds of M. cochinchinensis as described previously (19). The mass of the purified peptide was analyzed by electrospray ionization mass spectrometry, and purity was assessed by analytical RP-HPLC.

**Synthesis of MCoTI-II**—MCoTI-II was synthesized using native chemical ligation with methods established previously for disulfide-rich cyclic peptides (24–26).

**NMR Spectroscopy**—T1 and heteronuclear NOE experiments on free MCoTI-II (6 mM in 100% D2O) were recorded at 293 K on 500 and 600 MHz Bruker spectrometers using pulse programs based on those described by Farrow et al. (27). A recycle delay of 2 s was used, and for the T1 experiments, variable relaxation delays of 10, 20, 40, 50, 75, 100, 200, 300, 400, 500, 700, 1000, 1250, and 1300 ms were used. The data were analyzed with Tosspin (Bruker), and the experimental 13Ca T1 and NOE data were fitted using the model-free approach (28). The fit of the experimental and calculated T1 and NOE values was assessed using an r.m.s.$^7$ analysis (29).

$$r.m.s. = \sqrt{\frac{1}{4} \sum (W \text{exp} - W \text{calc})^2}$$

Here, the experimental values are denoted as “exp,” and the calculated values are denoted as “calc.” The final r.m.s. was weighted toward the T1 fit, with $W = 1$ for T1 values and $W = 0.5$ for NOEs, as the T1 is more accurate than the NOE (29). The overall r.m.s. for the model-free fit was 1.97%. Based on the model-free analysis, the average overall correlation time $\tau_0$ was calculated to be $2.4 \pm 0.05$ ns, the average internal correlation time $\tau_i$ was $20 \pm 0.4$ ps, and the overall average of the order parameters $S^2$ was calculated to be $0.84 \pm 0.02$. The two parameters used to describe the internal dynamics, $\tau_i$ and $S^2$, were recalculated with the overall correlation time fixed to 2.4 ns for all data points. The resulting average $\tau_i$ and $S^2$ were $22 \pm 0.8$ ps and $0.84 \pm 0.03$, respectively, and the overall r.m.s. value was 3.66%. All experimental and calculated T1 and NOE values are shown in Table 1 and Fig. 2.

**Analytical HPLC and LC-MS**—A solution of 10:1 MCoTI-II/trypsin (0.2 mM MCoTI-II and 0.02 mM L-tosylamido-2-phenylethyl chloromethyl ketone-treated bovine pancreatic trypsin (Sigma Aldrich)) in 100 µl of 10 mM KH2PO4, pH 7, was incubated at room temperature for 1 week. The stability of MCoTI-II in the presence of trypsin was monitored by analytical HPLC and LC-MS after 16, 40, 64, 112, and 164 h. At each time point, 20 µl of the reaction mixture was quenched with 4 µl of 4% TFA. The samples were immediately analyzed by analytical HPLC and LC-MS.

**Purification of MCoTI-II Trypsin Complex**—A complex of MCoTI-II bound to L-tosylamido-2-phenylethyl chloromethyl keton...
ethyl ketone-treated bovine pancreatic trypsin (Sigma Aldrich) was purified by cation exchange chromatography at 4 °C using FPLC (GE Healthcare). MCoTI-II and trypsin were combined in 50 mM NaCl, 50 mM Tris, pH 8, at a molar ratio of 1:2. The trypsin/MCoTI-II complex was loaded onto a 1-ml HiTrap SP HP column (GE Healthcare) and eluted using a 0.5%/min gradient of 0–20% 1M NaCl, 50 mM Tris, pH 8. The absorbance of the eluted complex was monitored at 280 nm, and mass and purity were evaluated by MALDI-TOF mass spectrometry and SDS-PAGE, respectively. FPLC-purified trypsin-MCoTI-II was concentrated in an Amicon Ultra 4 centrifugal filter device with a 5-kDa cut-off. The final protein concentration of 77 mg/ml was measured at 280 nm on a NanoDrop ND-1000 using an extinction coefficient of 37,650 M⁻¹ cm⁻¹.

Crystal Structure Determination of the MCoTI-II Trypsin Complex—Crystals were grown at 4°C by hanging drop vapor diffusion in drops composed of 1-μl well condition and 1 μl 23 mg/ml trypsin-MCoTI-II complex in 24-well plates containing shallow gradients of PEG 3350 and ammonium acetate in 0.1M BisTris, pH 6.5, buffer. The buffer used for crystallization was 15 mM Tris, pH 8.0, containing 30 mM NaCl, and crystals grown with 28% PEG 3350 and 0.24 M ammonium acetate in 0.1 M BisTris, pH 6.5, buffer as the well solution were selected for data collection and cryoprotected by the addition of ethylene glycol to 20% (v/v). Data were measured on beamline MX1 (30) of the Australian Synchrotron (wavelength, 0.953645 Å). Data were processed using HKL2000 (31), and the structure was solved by molecular replacement with Phaser (32) from the structure of bovine trypsin (Protein Data Bank code 2UUY (33)). Refinement was performed with Phenix (34), and model building was performed with COOT (35). Statistics are given in Table 2.

RESULTS

Synthesis and Characterization of MCoTI-II—MCoTI-II was synthesized using native chemical ligation (36) with methods established previously for other cyclotides (26). Cyclization and oxidation were achieved in a single step in 0.1 M ammonium bicarbonate, pH 8. Purified samples were analyzed by RP-HPLC and electrospray ionization MS.

Analysis of Backbone Dynamics of MCoTI-II—To determine whether the disorder in the three-dimensional structure of MCoTI-II is a result of flexibility or simply a lack of distance restraints in the structure calculations, the backbone dynamics of MCoTI-II in solution were analyzed using NMR relaxation experiments. Natural abundance 13C T1 and NOE spectra were recorded at several field strengths on the synthetic version of MCoTI-II. In these spectra, the signals for Cys21 and Arg24 appear to be overlapped with Lys10 and Lys9, respectively, and hence, these residues were not part of the relaxation analysis, which included 23 non-Gly residues of MCoTI-II. To describe the MCoTI-II backbone flexibility, the 13C relaxation data were analyzed using the Lipari-Szabo model-free approach (37), in which the order parameter, S², reflects the nano- to picosecond dynamics of the α-carbon backbone. S² can be between 0 and 1, with a high value representing small amplitude internal motions and a low value reflecting larger amplitude internal motions.
**Structural Analysis of MCoTI-II**

**TABLE 1** Experimental $^{13}$C T$_1$, and NOE values for MCoTI-II at 500 and 600 MHz, and calculated $T_1$ and NOE values based on the Lipari-Szabo model-free approach

<table>
<thead>
<tr>
<th>Residue (NOE)</th>
<th>$T_1$ (s)</th>
<th>NOE</th>
<th>$T_1$ (s)</th>
<th>NOE</th>
<th>RMS</th>
<th>%</th>
<th>$S^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>A4</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>A5</td>
<td>0.32</td>
<td>0.32</td>
<td>0.32</td>
<td>0.32</td>
<td>0.32</td>
<td>0.32</td>
<td>0.32</td>
</tr>
<tr>
<td>K6</td>
<td>0.27</td>
<td>0.27</td>
<td>0.27</td>
<td>0.27</td>
<td>0.27</td>
<td>0.27</td>
<td>0.27</td>
</tr>
<tr>
<td>L8</td>
<td>0.30</td>
<td>0.30</td>
<td>0.30</td>
<td>0.30</td>
<td>0.30</td>
<td>0.30</td>
<td>0.30</td>
</tr>
<tr>
<td>L9/C11</td>
<td>0.28</td>
<td>0.28</td>
<td>0.28</td>
<td>0.28</td>
<td>0.28</td>
<td>0.28</td>
<td>0.28</td>
</tr>
<tr>
<td>H10/H14</td>
<td>0.28</td>
<td>0.28</td>
<td>0.28</td>
<td>0.28</td>
<td>0.28</td>
<td>0.28</td>
<td>0.28</td>
</tr>
<tr>
<td>H11</td>
<td>0.28</td>
<td>0.28</td>
<td>0.28</td>
<td>0.28</td>
<td>0.28</td>
<td>0.28</td>
<td>0.28</td>
</tr>
</tbody>
</table>

The $T_1$ and NOE data for MCoTI-II are given in Table 1 and Fig. 2. The $^{13}$C-1H HSQC NMR spectrum of MCoTI-II and $S^2$ values for the intercysteine loops and individual residues are shown in Fig. 3. The overall correlation time for MCoTI-II is $2.4 \pm 0.2$ ns, and the average order parameter is $0.84 \pm 0.06$. Loop 6, with a significantly lower order parameter than the other loops ($S^2 = 0.76$) is more flexible in solution (Fig. 3). In addition, the order parameter for loop 1 ($S^2 = 0.79$, excluding residues 9 and 10) is smaller than the average order parameter, indicating that this loop is more flexible than other parts of the molecule. Interestingly, the residues on each side of Lys$^6$, the active site residue for trypsin inhibitory activity, have order parameters $\sim 0.1$ lower than the rest of the loop, with Pro$^5$ and Ile$^7$ at $S^2 = 0.75$. Loops 2, 3 and 4 have order parameters ($S^2 = 0.84$, 0.85, and 0.85, respectively) close to the average order parameter. Loop 5 appears to be the most ordered loop with $S^2 = 0.88$. This high value reflects the conformation of loop 5, which comprises a β-hairpin, consistent with a lack of flexibility in this loop. The cysteine residues have a considerably higher average order parameter ($S^2 = 0.90$) than the rest of the molecule, indicating that the core of the cystine knot motif is the most rigid part of MCoTI-II.

**DISCUSSION**

In this study, we analyzed the dynamics of MCoTI-II, a promising scaffold in drug design, and determined its structure bound to trypsin. Despite flexibility in loops 1 and 6 in solution, a well defined complex is formed upon binding to trypsin.
### TABLE 2

**Data collection and refinement statistics**

<table>
<thead>
<tr>
<th>Data collection</th>
<th>C2</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of trypsin complexes/a.u</td>
<td>3</td>
</tr>
<tr>
<td>Space group</td>
<td>C2</td>
</tr>
<tr>
<td>Cell dimensions</td>
<td>a = 136.1, b = 71.9, and c = 108.5 Å; α = 90.0, β = 119.8, and γ = 90.0°</td>
</tr>
<tr>
<td>Resolution (Å)</td>
<td>3.69–1.80/3.69–1.80</td>
</tr>
<tr>
<td>Rwork/Rfree</td>
<td>0.103 (0.449)/0.103 (0.449)</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>98.7 (98.7)</td>
</tr>
<tr>
<td>Redundancy</td>
<td>3.7 (3.7)</td>
</tr>
</tbody>
</table>

| Refinement | |
| No. of reflections | 78,423/4,122 |
| Resolution (Å) | 1.8 |
| Rwork/Rfree | 0.157 (0.220)/0.193 (0.246) |
| No. of atoms | |
| Protein | 5,677 |
| Water | 1,233 |
| Other | 11 |
| Ramachandran | |
| Favored | 97.4% |
| Outliers | 0.0% |
| r.m.s. deviations | |
| Bond lengths (Å) | 0.006 |
| Bond angles | 1.02° |

a.u., asymmetric unit.

* Data for highest resolution shell are shown in parentheses.

$R_{free}$ was calculated from 5% of reflections.

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**FIGURE 3.** 13C NMR relaxation analysis of MCoTI-II. **A**, 13C-HSQC spectrum of MCoTI-II with chemical shift assignments of the CoHa backbone; glycine residues are not shown. (The unlabeled peak is a CB from Ser15.) **B**, average order parameters for loop 1–6 (gray) and the cysteine residues in the cystine knot (black) of MCoTI-II. **C**, order parameters for each assigned residue. Residues in the loops are shown in gray, and the cysteine residues are shown in black. The dashed line indicates the overlapped Cys11 residue that was not included in the analysis.

**FIGURE 4.** Three-dimensional structure of MCoTI-II bound to bovine trypsin. **A**, MCoTI-II is shown in magenta, and trypsin is shown in gray. **B**, the active site lysine residue is highlighted in blue. **C**, the crystallographic B-factors are mapped onto the MCoTI-II structure. The thicker lines represent higher B-factors. **D**, a superposition of the bound (magenta) and free (green) structures of MCoTI-II. The positions of the intercysteine loops are labeled with Arabic numbers in B–D. The coordinates for the complex of MCoTI-II bound to trypsin have been deposited in the Protein Data Bank code 4GUX.
Structural Analysis of MCoTI-II

Unexpectedly, the cyclization loop is involved in the interaction with trypsin, providing an explanation for the highly potent subnanomolar inhibitory activity observed for MCoTI-II (21). The similarity between the crystal structure and solution structures of MCoTI-II suggests that no major conformational changes occur on binding, but there are minor local changes, including the selection of a specific conformation of the active site Lys residue in the complex. Analysis of the dynamics of MCoTI-II free in solution suggests that the flexibility observed for the residues adjacent to the active site residue and for residues in loop 6 might contribute to the peptide being able to adopt a conformation allowing optimal binding.

The flexibility observed for the cyclization loop in MCoTI-II is consistent with the presence of several small amino acids such as glycine and serine and is also consistent with the dynamics analysis of MCoTI-I in solution (20). Despite this localized flexibility in solution, MCoTI-II is very stable in human serum, as is the case for other cyclotides such as kalata B1 (11). Presumably, the well-defined structural core, conferred primarily by the cystine knot motif, is sufficient to prevent degradation by proteases. The NMR relaxation data reported here unequivocally demonstrate the rigidity of the cystine knot core.

Although cyclization is thought to have evolved primarily because of advantages conferred by enhanced structural stability, this study demonstrates that another benefit of cyclization is that it creates an additional structural loop that can be involved in target interactions as shown in Fig. 6. These additional interactions probably explain the enhanced trypsin inhibitory potency of the cyclic peptide compared with linear forms; MCoTI-II has a $K_i$ of 0.03 nM compared with 0.3 nM for a version devoid of loop 6 (21). A role in target interaction has previously been postulated for the cyclic backbone of MCoTI-II (40), but here, we provide the first experimental evidence to support this suggestion. The detailed structural information regarding the binding of MCoTI-II to trypsin should be valuable for subsequent modeling and the design of novel peptides with potential therapeutic activities, particularly for the design of novel enzyme inhibitors. Such modeling and design approaches have already been proven successful for modifying the smaller cyclic trypsin inhibitor sunflower trypsin inhibitor-1 to target kallikrein-4, a protease overexpressed in prostate cancers (41).

The crystal structure of MCoTI-II bound to trypsin appears to be inconsistent in several ways, with the surprising recent report that MCoTI-I apparently becomes more flexible upon binding to trypsin than it is in solution (20). That study originally reported a generalized increase in flexibility of the active site loop (loop 1) of MCoTI-I on binding to trypsin but was modified in a corrigendum (where an error in NMR data analysis was corrected) to conclude that only five residues have increased flexibility upon binding to trypsin, including the active site Lys residue. This interpretation seems to be questionable, given that the side chain amino group of the active site residue in MCoTI-I is clearly detectable in the HSQC spectrum of the complex. Conformationally exchanging or solvent-exposed primary amino groups are not normally visible in NMR spectra due to exchange, so the clear appearance of the Lys amino group in the spectrum is consistent with protection from solvent (19) and a well-defined ($i.e.$ not flexible) bound conformation. Furthermore, the other putatively flexible residues are very well defined in the complex of MCoTI-II with trypsin and are involved in either intra- or intermolecular hydrogen bonds. Finally, given that MCoTI-I and II differ by only two residues and as we show that there is a well-defined complex between...
MCoTI-II and trypsin, it seems highly unusual that the active site residue in MCoTI-I would have enhanced flexibility upon binding to trypsin. Indeed, we propose here that this enhanced flexibility is not intrinsic to the binding process but reflects flexibility in a cleaved product. We found that MCoTI-II bound to trypsin is partially cleaved over a period of days; it is likely that MCoTI-I is also cleaved over a similar time scale, and the presence of an undetected open form of MCoTI-I in the Puttamadappa et al. (20) study could provide an explanation for the apparent flexibility of some residues, as the termini of the open form are likely to be highly flexible. Further study is required to confirm if this is the reason for the discrepancy with the previously published work on MCoTI-I.

Analysis of the structure of MCoTI-II bound to trypsin reported here also provides significant new insights and a different interpretation of the chemical shift changes observed for MCoTI-I upon binding to trypsin to that reported earlier (20). Several chemical shift changes in MCoTI-I were observed upon binding to trypsin. For instance, the chemical shift of the amide proton of Ala20 in loop 3 changes by 0.45 ppm upon binding with trypsin (20) and that of Cys17 changes by 0.46 ppm. Our crystal structure reveals that several MCoTI-II residues are involved in binding with trypsin (Fig. 6, A and B) but that Ala20 forms an intramolecular hydrogen bond with Val3, as shown in Fig. 6C, and Asp16 forms a hydrogen bond with Lys9 (Fig. 6D). Therefore, although these chemical shift changes were interpreted (20) to be associated with direct interactions with trypsin, our study indicates that they are actually the result of intramolecular structural changes at sites either involving or adjacent to these residues and are not directly a consequence of interaction with trypsin.

In summary, we report the structure of a cyclotide bound to a protein target and provide mechanistic insights into the biological role of the cyclic backbone. We show that MCoTI-II forms a tight complex with trypsin and that the flexibility in the solution state around the active site residue and throughout cyclization, loop 6, relative to the rest of the molecule, allows the peptide to modify its conformation in these regions upon binding to trypsin to maximize favorable interactions. These interactions provide an explanation for the high potency of MCoTI-II. Interestingly, backbone cyclization appears to not only assist in the stabilization of proteins but also facilitates extra binding interactions that can enhance potency. Naturally occurring cyclic proteins such as cyclotides have significant potential as templates in drug design and, in particular, MCoTI-II has been used as a scaffold in the design of a range of novel protease inhibitors. The data obtained from the current study that define the interactions of MCoTI-II with a serine protease might therefore be useful in the design of more potent inhibitors and in understanding how to tailor the specificity of this inhibitor to other related proteases of pharmaceutical interest.

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REFERENCES


Structural Analysis of MCoTI-II


