Structure-oriented rational design of chymotrypsin inhibitor models

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Three peptides modelling a highly potent, 35-residue chymotrypsin inhibitor (Schistocerca gregaria chymotrypsin inhibitor) were designed and synthesized by convergent peptide synthesis. For each model peptide, the inhibitory constant (Ki) on chymotrypsin and the solution structure were determined. In addition, molecular dynamics calculations were performed for all of them. Two models containing approximately half of the parent inhibitor (17 of 35 residues) were designed and subsequently found to have no substantial inhibitory activity (Ki values in the mM range). The third model composed of 24 amino acid residues proved to be an effective (Ki = 10⁻⁷) inhibitor of bovine chymotrypsin. Both the solution structure properties determined by NMR spectroscopy and the dynamic behaviour of the latter model system are comparable to the native inhibitor. In contrast, the structure and dynamics of the first two related model peptides show characteristic differences. We suggest that the conformation and flexibility of the modelled protease inhibitor are crucial for its biological efficiency. Moreover, the structural and dynamic features of the binding loop (28–33) and those of the rest of the molecule appear to be interdependent. Most importantly, these structural characteristics can be rationally modified, at least partially, by peptide design.

Keywords: molecular modelling and dynamics/NMR spectroscopy/peptide design/protease inhibition/structure–activity relationship

Introduction

Biological background and significance

Unanswered questions concerning the fine details of the structure–activity relationship of canonical serine protease inhibitors still remain. Canonical, standard mechanism serine protease inhibitors can be classified into numerous structural families with different overall folds (Bode and Huber, 1991). Canonical inhibitors bind in the active cleft of the enzyme in a substrate-like manner (Bode and Huber, 2000). Unlike non-proteic inhibitors, these molecules possess all functional groups required for proper hydrolysis, thus the apparent paradox, namely that they act as inhibitors, remains to be explained. The key to inhibition is the extremely slow rate of hydrolysis of the P1–P1' peptide bond, which is undoubtedly a consequence of the three-dimensional structure and dynamics of the binding loop and its spatial neighbourhood (Laskowski and Qasim, 2000; Ravichandran et al., 2001; Cai et al., 2002).

In this paper, we focus on the 35-residue peptide SGCI (Schistocerca gregaria chymotrypsin inhibitor), an inhibitor of the newly established ‘grasshopper family’ (Laskowski and Qasim, 2000), recently named also ‘pacifastin family’ (Simonet et al., 2002). SGCI and its close homologue SGTI (Schistocerca gregaria trypsin inhibitor) were first isolated in 1998 from desert locusts (Hamdaoui et al., 1998). SGCI is an outstanding chymotrypsin inhibitor [Ki (bovine, pH 8.0, 25°C) = 6.2×10⁻¹² mol dm⁻³], whereas SGTI is a good inhibitor for trypsin [Ki (bovine, pH 8.0, 25°C) = 2.1×10⁻⁷ mol dm⁻³] and an average inhibitor for chymotrypsin [Ki (bovine, pH 8.0, 25°C) = 2.0×10⁻⁶ mol dm⁻³] (Malik et al., 1999). Related peptides, designated PMP-C and PMP-D2, were obtained from the migratory locust, Locusta migratoria, in the early 1990s (Boigeaian et al., 1992). Sequence alignment (Figure 1) reveals that SGCI is an orthologue of PMP-C whereas SGTI is an orthologue of PMP-D2 (for a recent review on the inhibitor family, see Simonet et al. (Simonet et al., 2002)).

The solution structure of PMP-D2 (Mer et al., 1994), PMP-C (Mer et al., 1996a), SGCI and SGTI (Gáspári et al., 2002) was determined by NMR spectroscopy. The crystal structure of PMP-D2 and PMP-C complexed with chymotrypsin was solved recently (Roussel et al., 2001). All these peptides share a common fold characterized by three antiparallel β-pleated sheets and three conserved disulfide bridges. The protease binding loop is located near the C-terminus of the inhibitors in the spatial neighbourhood of conserved residues. Natural PMP-C and SGCI are fucosylated at Thr9 (Mer et al., 1996b). This modification was suggested to contribute to the structural stability of PMP-C (Mer et al., 1996a), although the non-fucosylated form of both inhibitors retains full activity (Mer et al., 1996b). The internal dynamics of PMP-D2 were studied by ¹³C relaxation and molecular dynamics methods and the authors concluded that there are slow conformational motions in this peptide (Mer et al., 1996c).

Structure of SGCI

The overall fold of SGCI, determined in our laboratory (Figure 2), corresponds to the traditional structure characteristic of the grasshopper inhibitor family (Gáspári et al., 2002). The molecule contains three slightly twisted antiparallel β-sheets (spanning residues 9–10, 16–19 and 26–28) and three disulfide bonds (Cys4–Cys19, Cys17–Cys28 and Cys14–Cys33). Residues 5–8 form a type II β-turn. In SGCI, the P1–P1'
Investigated by NMR (Shaw et al., 1998). Structures of canonical inhibitors whose side chain dynamics were studied were represented by a relatively high backbone r.m.s.d. value (0.78 Å), indicating that this region is not as well defined as the core of the molecule and, possibly, exhibits a greater degree of flexibility. This is not an uncommon finding among canonical inhibitors whose structure and dynamics were investigated by NMR (Shaw et al., 1995; Liu et al., 1996, 1998).

Interestingly, the protease binding loop (residues 28–33) is represented by a relatively high backbone r.m.s.d. value (0.78 ± 0.23 Å), indicating that this region is not as well defined as the core of the molecule and, possibly, exhibits a greater degree of flexibility. This is not an uncommon finding among canonical inhibitors whose structure and dynamics were investigated by NMR (Shaw et al., 1995; Liu et al., 1996, 1998).

Design and synthesis of inhibitor models

Although the sequence of the P1–P1' (Leu30–Lys31) region in SGCI resembles a good substrate for chymotrypsin, SGCI is actually a highly potent inhibitor of chymotrypsin. The solution of this apparent contradiction lies in the three-dimensional structure of SGCI, concerning especially the conformation and dynamics of the protease binding loop (Laskowski and Qasim, 2000).

The goal of the present study was to find the ‘minimal’ structural parts of SGCI responsible for inhibition. SGCI was chosen as a model because of its small size and compact structure. We assumed that retaining the protease binding loop with the P1–P1’ site and the residues located in its close sequential and structural neighbourhood (as a minimum, residues 28–33 and 13–16), the inhibitory activity can be more or less retained, even on removing the ‘inactive parts’. In contrast to alanine scan and other ‘linear’ sequence-trimming and residue-substitution approaches, our concept is entirely conformation-based. We found only a limited number of attempts described in the literature using conceptionally comparable approaches to investigate structure–activity relationships of protease inhibitors. Studies using interleukin 1β as a scaffold for the reactive site loop of α1 antitrypsin (Wolfson et al., 1991; Arico-Muendel et al., 1999) and investigations with cyclic nonapeptides corresponding to the protease-binding part of a Bowman–Birk inhibitor (Terada et al., 1980; Dittmann et al., 2001) should be mentioned here.

To test our hypothesis, three model peptides, denoted MP1, MP2 (17 residues) and MP3 (24 residues), containing selected regions of SGCI were designed (Figure 3) [details of their synthesis were described by Mucsi et al. (Mucsi et al., 2002)]. As these regions are not formed by a continuous polypeptide chain but rather by residues located in distant parts of the original sequence, we applied chemical modifications such as cyclization and isopeptide bond formation (i.e. βAsp-NH- and γGlu-NH-) to keep the conformation of the selected parts native-like. In order to retain the original β-sheet structures in the model, of reduced peptide main chain length, antiparallel β-sheets B1 and B2 were closed by a flexible Gly–Gly ‘bridge’ to form a cyclopeptide ring of either 10 or 14 amino acid residues. The other fragment composed from 7 or 10 amino acid residues was linked to the cyclopeptide ring as a ‘tail-peptide’. In order to simplify the synthesis, selective disulfide bridge formation was avoided. (The disulfide bonds omitted or replaced were assumed to contribute to the stabilization of the structure rather than being involved in enzyme–inhibitor interactions.) For this reason, one of the cysteine residues (Cys17 in MP1 and MP2,
and Cys19 in MP3) was replaced by glutamic acid and the \( \gamma \)-carboxyl group of the latter was condensed with the N-terminal amino group of the tail peptide, to form an amide bond instead of the disulfide bridge.

**Evaluation of the activity and structure of the model peptides**

In each model, the effectiveness of preserving the molecular structure by both the Gly–Gly ‘bridge’ and the side-chain \( \gamma \)-peptide bond was checked by short molecular dynamics (MD) runs using SYBYL (Tripos, 2001). Initially, two models were chosen on the basis of comparisons of preliminary MD properties of the model peptides and SGCI. MP1 was constructed tentatively to behave as an inefficient inhibitor model within a native-like SGCI scaffold. Owing to the drastic structural change, the aromatic side chain of residue 10 was expected to be recognizable by chymotrypsin and hence the cleavage of the Phe10–Lys11 bond should lead to degradation of the inhibitor, regardless of the extent of enzyme–inhibitor–binding loop interaction. To avoid this effect associated with the native-like amino acid sequence, MP2 was constructed in which Phe10 is replaced by Thr. Furthermore, we presumed that a substrate may turn into an inhibitor if the conformation and the dynamic properties of the molecule are properly modified. Thus MP3, incorporating more residues from the parent molecule SGCI than the two previous model peptides MP1 and MP2, was designed to behave as a medium-range inhibitor of chymotrypsin.

In order to test the above-mentioned hypothesis experimentally, the three selected model peptides were prepared and their inhibitory constants were determined by standard methods. The solution structure of all three model peptides was determined by homonuclear \( ^1 \)H NMR spectroscopy. Molecular dynamics simulations (both in vacuum and in aqueous solution) were also performed both for SGCI and for MP1–3.

**Materials and methods**

**Materials**

Model peptides 1–3 (MP1–3) were prepared as described previously (Mucsi et al., 2002). Sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) was obtained from Cambridge Isotope Laboratories (Andover, MA), Bovine chymotrypsin, \( N \)-succinyl-Ala–Ala–Pro–Phe-\( p \)-nitroanilide, TRIS and Triton X-100 were purchased from Bachem (Bubendorf, Switzerland).

**Synthesis**

All three models were synthesized using convergent peptide synthesis (Riniker et al., 1993) as described earlier (Mucsi
Table I. Inhibition activities of model peptides MP1–3 and SGCI against bovine chymotrypsin

<table>
<thead>
<tr>
<th>Peptide</th>
<th>$K_i$ (mol dm$^{-3}$)</th>
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<tbody>
<tr>
<td>MP1</td>
<td>$2.24 \times 10^{-4}$</td>
</tr>
<tr>
<td>MP2</td>
<td>$3.70 \times 10^{-3}$</td>
</tr>
<tr>
<td>MP3</td>
<td>$3.00 \times 10^{-2}$</td>
</tr>
<tr>
<td>SGCI</td>
<td>$6.20 \times 10^{-12}$</td>
</tr>
</tbody>
</table>

et al., 2002). The tail peptides (Thr29–Gln35 or Ala26–Gln35 in SGCI) and the cyclopeptide part (residues 10–17 or 8–19 closed by a Gly–Gly bridge, according to the numbering of SGCI) of molecules (Figure 3) were synthesized separately. Starting peptide fragments were prepared by the Fmoc (fluorenylloxycarbonyl) technique in the solid phase (Atherton and Sheppard, 1989). Cyclization was carried out with BOP [benzotriazol-1-yl-oxytris(dimethylamino)phosphonium hexafluorophosphate] reagent (Atherton and Sheppard, 1989) in dilute DMF ($N$-$N$-dimethylformamide) solution containing NMM ($N$-methylmorpholine), Boc ($tert$-butoxycarbonyl) and $i$-Bu ($tert$-butoxy) protecting groups were cleaved by TFA (trifluoroacetic acid). The protecting groups were cleaved by HF containing anisole at –5°C. The disulfide bridge was built up in MP1 and MP2 by iodine in HCl–acetic acid solution and in MP3 by oxygen in buffer solution (Andreu et al., 1994). The purities of the peptide fragments and target molecules were checked by RP-HPLC. The final products and the intermediates were purified by HPLC and their structures were characterized by amino acid analysis and mass spectrometry.

**Enzyme inhibition**

Inhibitory activities were measured on a Shimadzu UV-210PC spectrophotometer (Shimadzu, Kyoto, Japan) at 410 nm using 3×3 mm quartz cuvettes. The reaction mixture contained the following materials: 0.1 M NaCl, 0.05 M TRIS buffer (maintained at pH 8.0), 0.01 M CaCl$_2$ and 0.005% Triton X-100 as emulsifier. As substrate, $N$-succinyl-Ala–Ala–Pro–Phe-$p$-nitroanilide (0.1 mM) was used. The selected model peptide was added in various concentrations ranging from 8.62 to 34.5 pM. The concentration of bovine chymotrypsin was set to 0.3 μM. The absorption record was analysed using the Progress data analysis program (Leatherbarrow, 1987) to determine the inhibitory constant $K_i$ using Equation 1 by a non-linear fitting method (Dixon, 1972; Bieth, 1995):

$$\frac{[E]}{[E_0]} = 1 - \frac{[E_0] + [I_0] + K_i^*}{\sqrt{(E_0) + [I_0] + K_i^*}^2 - 4[E_0][I_0]}$$

where $[E_0]$ and $[E]$ are the starting and actual concentrations, respectively, $[I_0]$ is the initial concentration of the inhibitors or model peptides and $K_i^*$ is the apparent value from which $K_i$ is calculated by the equation

$$K_i = \frac{K_i^*}{1 + [S]}$$

where $[S]$ is the concentration of the substrate and $K_m$ is its Michaelis–Menten constant. If necessary, the hydrolysis of the model peptides was monitored by using an ABI 471A amino acid sequencer.

**NMR spectroscopy**

The 1D and 2D homonuclear $^1$H NMR spectra of all three model peptides (1.5 mg) dissolved in H$_2$O–D$_2$O (9:1 v/v) were recorded at 297 K on a Bruker DRX 500 spectrometer. In the direct dimension the number of acquired data points was typically 2048 with 512 increments. Spin lock of ~60 ms and a mixing time of 150 ms were used for TOCSY and NOESY measurements, respectively. Data processing in the $t_2$ dimension was carried out by zero filling up to 4096 points and applying a Kaiser window function. In the indirect dimension, zero filling up to 1024 points was applied and a shifted sine-bell window function was used. Spectra were referenced to internal DSS. For spectrum analysis, the triad module of the software SYBYL (Tripos, 2001), running on an SGI R12000 workstation, was used. Resonance assignment was carried out according to the common procedure described in the literature (Redfield, 1993).

**Structure calculations**

Structure calculations were based on NOE-derived distance restraints which were obtained from integration of the NOESY cross-peaks by using the module triad of the program SYBYL (Tripos, 2001). Based on the relative size of the integrals, restraints were classified into three categories corresponding to distance ranges of 1.8–2.5, 1.8–3.5 and 1.8–5.0 Å. The 1.8 Å value was chosen as a good approximation of the sum of the van der Waals radii of two geminal but non-overlapping hydrogen atoms (e.g. H$\alpha_1$ and H$\alpha_2$ of a G1 residue).

Structure calculations were carried out with the program X-PLOR 3.851 by using standard simulated annealing protocols (Nilges et al., 1988, 1991). High-temperature calculations were run at 1000 K for 3000 steps followed by 3000 cooling steps with the NOE scale parameter set to 50. For each molecule a family of 100 structures were calculated. For comparison, 10 structures were accepted with good geometry parameters, in which no NOE violations >0.3 Å occurred.

**Molecular dynamics calculations**

MD calculations for synthetic model peptides MP1–3 and SGCI were carried out both in vacuum (Procedure A) and in explicit aqueous solution (Procedure B, similar to the approach described by Li and Daggett (Li and Daggett, 1995)) using the program package GROMACS 3.0.2 (Lindahl et al., 2001) on a 450 MHz Pentium II PC running Linux. In both cases all Asp and Glu residues were considered as negatively charged and Lys and Arg side chains as protonated. For each synthetic model peptide and SGCI, the average NMR structure was chosen as the starting conformation. All minimizations were carried out with the steepest descent method.

**Procedure A.** Models were first minimized to the rather small 500 kJ mol$^{-1}$ nm$^{-1}$ force limit. MD simulation was run for 10 ns (5×10$^6$ steps) at 300 K by using a time step of 2 fs. Structures were saved every 0.5 ps for further analysis.

**Procedure B.** After having minimized to the 500 kJ mol$^{-1}$ nm$^{-1}$ force limit, the peptide (at neutral pH) was surrounded by water molecules. Simulations were run in a rectangular box, with walls at least 0.5 nm away from any of the peptide atoms. Energy minimizations were carried out first with all water moieties and subsequently with all peptide atoms frozen, to the 500 kJ mol$^{-1}$ nm$^{-1}$ force limit. To ensure that the water molecules filled the simulation box smoothly, MD calculations...
were run only on the water molecules for 500 ps (2.5×10⁵ steps). Subsequently, water molecules were minimized once again; thereafter, the whole system was minimized. The full MD simulation with no constraints was run for 1 ns (5×10⁵ steps) with a 2 fs time step at 300 K. For analysis, structures were saved every 0.5 ps.

**Results**

*Inhibition of chymotrypsin by the model peptides*

Biological studies revealed that MP1 has no significant inhibitory activity on chymotrypsin ($K_i = 2.24 \times 10^{-4}$ mol dm⁻³; Table I). Our results indicate that MP1 contains two cleavage sites for chymotrypsin: additionally to the P₁–P₁’ (Leu30–Lys31) site, the Phe10–Lys11 bond is also hydrolyzed. MP2 derived from MP1 by replacing Phe10 with Thr also does not inhibit chymotrypsin ($K_i = 3.70 \times 10^{-2}$ mol dm⁻³; Table I). For this molecule, no proteolytic cleavage other than that at the P₁–P₁’ bond was observed.

In MP3, the critical Phe10 was replaced by chymotrypsin-resistant Ile10 because the latter residue is ready to form a β-sheet motif and may also substitute Phe10 with respect to its hydrophobic core-forming ability. Consistent with our expectations, MP3 proved to be an effective inhibitor of bovine chymotrypsin ($K_i = 3.0 \times 10^{-7}$ mol dm⁻³), although not completely comparable to the wild-type SGCI (Table I). However, the $K_i$ value measured for MP3 is not only higher than the limit accepted for inhibitory activity but is almost equal to that obtained for SGTI, a natural inhibitor ($K_i = 2.1 \times 10^{-7}$ mol dm⁻³) for trypsin. In the case of MP3, in which the P₁–P₁’ sequence is conserved, the enhanced steric cooperativity, i.e. the decreased mobility and a more rigid binding loop, are able to convert a substrate into an inhibitor.

Because steric factors including 3D structure and conformational motions seem to be important in determining the substrate or inhibitory character, we investigated the solution structure and MD properties of MP1–3 by NMR spectroscopy and MD simulations.

*Resonance assignment of the NMR spectra*

The assignment of chemical shifts was straightforward for all three molecules. All residues could be unambiguously identified from the homonuclear spectra.

NH and Hα chemical shift values are indicative of the chemical and structural similarity of the model peptides and SGCI. Hα chemical shifts are more suitable for such comparison than NH shifts because of their weaker pH dependence. Only chemical shift values of residues ‘conserved’ in all three
model peptides and those of SGCI can be compared directly (Figure 4).

The \( \delta \alpha \) chemical shifts of MP1 and MP2 are very similar, indicating related conformations. In contrast, the chemical shift values of MP3 deviate from those of both MP1/MP2 and SGCI. The greatest difference in \( NH \) chemical shifts was observed for Glu19, Cys17 and Cys19, as these residues (and their chemical environment) were altered mostly in this model peptide. Large differences in \( \Delta \delta NH \) and \( \Delta \delta \alpha \) values were also found for Lys13 too, but the \( \Delta \delta \alpha \) values for this residue are not significant. The unusual values of \( \delta NH \) and \( \delta \alpha \) found for Lys13 in SGCI may be indicative of a structurally important, uncommon backbone conformation in the middle of the backbone (Lys11–Asp15) located close to the protease binding loop. In contrast to Lys13, Lys31 exhibits the usual chemical shift values in SGCI and the \( \Delta \delta NH \) and \( \Delta \delta \alpha \) values of this residue obtained for SGCI and MP3 are small. This fact possibly reflects that in MP3 the structure of Lys31, the PI’ residue in the binding loop, is close to its native conformation observed in SGCI.

Solution structure of the model peptides

The solution structure of each model peptide was determined by homonuclear \(^1H\) NMR spectroscopy. MP1 has no well-defined structure (Figure 5A) owing to the small number of NOE-derived distance restraints which could be used in the structure calculations (Table II). R.m.s.d. values of the 10 best structures of MP1 are shown in Table III. The binding loop also seems to be present in multiple conformations. As expected, the aromatic side chain of Phe10 is no longer buried in the molecule but exposed to solvent, which is in sharp contrast to what was found in native SGCI. The results obtained for MP2 are very similar to those for MP1. Similarly to MP1, we could not identify any traces of \( \beta \)-strands in MP2.

In contrast to MP1 and MP2, owing to the increased number of distance restraints (348), the structure of MP3 could be determined with acceptable accuracy. Its overall fold is reminiscent of that of SGCI (Figure 5C). The number of distance restraints (Table II) is sufficient to obtain a relatively well-defined ensemble of a single conformer. Although most r.m.s.d. values (Table III) are closer to those obtained for MP1 and MP2, the binding loop is much better defined and its r.m.s.d. (0.78 ± 0.23 Å) is comparable to that of found in SGCI (0.81 ± 0.23 Å). As in the former two model peptides, the Gly–Gly bridge and the \( \gamma \)-amide linkage between Glu19 and Ala26 are the least determined parts of the molecule.

In MP3, the NOE cross-peaks observed between the \( \alpha \) and \( \beta \) atoms of Cys residues allowed the identification of the disulfide bridges (Klaus et al., 1993), namely Cys17–Cys28 and Cys14–Cys33 (Mucsi et al., 2002). According to the NMR spectroscopic investigations, the most rigid parts of this model are the 11–16 loop and the binding loop (28–33), and the most mobile sequence is the Gly–Gly part. Although there are no segments in the molecule that can be unambiguously classified as \( \beta \)-sheets, the cyclopeptide adopts an elongated conformation indicative of interactions between segments Thr9–Lys11 and Thr16–Arg18, as confirmed by circular dichroism (CD).
Molecular dynamics simulations of the model peptides

To estimate the internal mobility of the inhibitor models, we performed long-time (10 ns) MD simulations in vacuum and short-time (1 ns) runs in water for each of the model peptides and SGCl. The starting conformer for simulations was chosen to be the one closest to the average structure obtained from the NMR structure calculations. To avoid misinterpretation of the observed conformational changes, the first 200 ps of the simulation time was excluded from the structural comparisons presented below.

The mobility of Cα atoms in the peptide backbone shows that MP1 and MP2 exhibit greater structural fluctuations than MP3, which is still less rigid than SGCl. This is most pronounced for regions corresponding to the β-strands (residues 9–11, 16–19 and 26–29) of native SGCl. The fluctuation of backbone torsion angles (φ, ψ) in the binding loop of the peptides indicates greater conformational flexibility in the case of MP1 and MP2 than for MP3 and the most constrained SGCl. Moreover, in SGCl and MP3, coordinated motion of the

Table V. Averages and deviations of φ and ψ torsion angles in binding loop of model peptides MP1–3 and SGCl (Gáspári et al., 2002)

<table>
<thead>
<tr>
<th></th>
<th>Thr29φ</th>
<th>Thr29ψ</th>
<th>Leu30φ</th>
<th>Leu30ψ</th>
<th>Lys31φ</th>
<th>Lys31ψ</th>
<th>Ala32φ</th>
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<tbody>
<tr>
<td>MP1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td>64.6</td>
<td>121.2</td>
<td>−135.2</td>
<td>58.4</td>
<td>−98.8</td>
<td>48.7</td>
<td>−127.5</td>
<td>148.0</td>
</tr>
<tr>
<td>Deviation</td>
<td>±83.5</td>
<td>±86.2</td>
<td>±78.6</td>
<td>±75.5</td>
<td>±65.7</td>
<td>±80.0</td>
<td>±49.1</td>
<td>±80.4</td>
</tr>
<tr>
<td>MP2</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td>−28.2</td>
<td>−136.7</td>
<td>−158.7</td>
<td>112.9</td>
<td>−80.9</td>
<td>−107.9</td>
<td>−121.7</td>
<td>114.3</td>
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<tr>
<td>Deviation</td>
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<td>±78.3</td>
<td>±65.1</td>
<td>±64.1</td>
<td>±60.7</td>
<td>±89.9</td>
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<tr>
<td>Average</td>
<td>−166.3</td>
<td>1.3</td>
<td>57.1</td>
<td>153.8</td>
<td>−58.4</td>
<td>−99.7</td>
<td>69.8</td>
<td>±176.7</td>
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<tr>
<td>Deviation</td>
<td>±36.1</td>
<td>±32.2</td>
<td>±24.5</td>
<td>±28.9</td>
<td>±15.8</td>
<td>±74.6</td>
<td>±61.0</td>
<td>±95.9</td>
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<td>SGCl</td>
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<tr>
<td>Average</td>
<td>−70.6</td>
<td>15.6</td>
<td>−11.8</td>
<td>118.2</td>
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<td>3.7</td>
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<td>±21.1</td>
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<td>±71.7</td>
<td>±45.3</td>
<td>±62.4</td>
<td>±52.6</td>
<td>±72.1</td>
<td>±68.2</td>
</tr>
</tbody>
</table>

'Typical'

|        |        |        |        |        |        |        |        |        |
| Average| −80    | 160    | −110   | 35     | −85    | 165    | −115   | 110    |

Torsion angles were calculated with MOLMOL (Koradi et al., 1996). In the last row the torsion angles of ‘typical’ canonical inhibitors are shown (Laskowski and Qasim, 2000).
binding loop and the segment opposite to it (residues 14–17) can be observed (Figure 6).

Discussion

Comparison of MP1 and MP2

On the basis of the inhibitory properties of MP1, it may be concluded that the peptide bond next to Phe10 is a suitable site for chymotrypsin, as speculated at the stage of peptide design. As structural data confirm, in MP1 the side chain of this residue is exposed to solvent, as many of the side chains interacting with Phe10 in SGCI are missing from MP1. In MP2, Phe10 was replaced by Thr. Thr frequently takes part in a β-sheet motif (Prevelige and Fasman, 1989) and is more polar than Phe with a β-branching side chain not preferred by the substrate-binding cleft of chymotrypsin. The fact that MP1 has a smaller $K_i$ than MP2 may be due to the second cleavage site (Phe10–Lys11) in the cyclopeptide part. We assume that the $K_i$ values of the cleavages are independent and we are measuring the $K_i$ value of the stronger complex. Thus the value $K_i = 10^{-4}$ mol dm$^{-3}$ is associated with the tighter complex actually formed at the second site (Phe10–Lys11) rather than at the P1–P1' site (Leu30–Lys31). This suggestion is consistent with other data in the literature: the $K_i$ inhibitory constant is decreased by two orders of magnitude when the P1 Leu residue is changed to Phe in some canonical inhibitors (Ardelt and Laskowski, 1991; Laskowski and Qasim, 2000). Additionally, substrates containing Phe at position P1 bind one or two orders of magnitude better to the enzyme than those with Leu in the same position (Antal et al., 2001).

While designing MP1 and MP2, based on preliminary structural studies, it was assumed that these two model peptides are too small to maintain the effective conformation and mobility of the binding loop. Our results obtained from MD simulations are in agreement with these expectations, as the relatively increased mobility of the backbone of the cyclopeptide (when compared with both MP3 and SGCI) is associated with different dynamics of the binding loop region.

Structure and activity of MP3

MP3 was designed to be a more effective inhibitor than MP1 and MP2. Therefore, both its cyclopeptide scaffold and its tail peptide which contains the binding loop residue are larger than in the other two model peptides. Furthermore, MP3 also contains the Cys28–Cys19 disulfide bond replaced by an isopeptide bond in the former two models MP1 and MP2. The critical residue Phe10 is replaced by Ile because of its higher β-sheet-forming potential compared with Thr (Prevelige and Fasman, 1989). On the other hand, the apolar Ile10 residue was expected to substitute Phe10 sufficiently well in organizing the interacting hydrophobic side chains.

Both the activity and the structural features of MP3 are closer to those of SGCI than MP1 and MP2. The well-defined structure of MP3 is comparable to that of SGCI, although there are still considerable differences. The lack of ‘genuine’ β-strands and the different relative orientation of the binding loop with respect to the rest of the molecule should be mentioned. However, the chemically modified scaffold of MP3 proved to be suitable for maintaining a biochemically efficient conformation of the protease binding loop. The importance of the proper scaffold is apparent when the ineffective peptides MP1 and MP2 are considered. The conformational and biochemical similarity of the model peptides to SGCI is interdependent, best exemplified by MP3, as its structural and dynamic properties appear to be suitable for inhibition.

Conclusions

The structure–activity relationships of the three model peptides of similar structure reveal the significance of the structure and dynamics of the binding loop of protease inhibitors. The importance of the conformation and flexibility of the protease binding loop has already been discussed in the literature (Laskowski and Qasim, 2000) and is continuously refined (Cai et al., 2002). However, to the best of our knowledge, our study is the first systematic investigation in the field using the approach of structure-oriented dissection of an inhibitor and reconstruction of the relevant parts with chemical synthesis to yield model peptides. Our results suggest that the scaffold ‘holding’ the binding loop (especially residues 13–16) plays an essential role in maintaining the proper conformation and mobility of the residues located in the reactive site. The test system that we have developed allows the adjustment of the structural features of this scaffold, the tuning of the inhibitory activity and the evaluation of the changes introduced. We suggest that similar approaches can effectively contribute to the understanding of serine protease inhibition and, more generally, to protein–protein interactions and can also be useful in rational molecule design.

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