Inhibition of human \( \mu \)-calpain by conformationally constrained calpastatin peptides

José Pfizer¹, Irmgard Assfalq-Machleidt², Werner Machleidt³ and Norbert Schaschke⁴,*

¹Max-Planck-Institut für Biochemie, Am Klopferspitz 18, D-82152 Martinsried, Germany
²Abteilung für Klinische Chemie und Klinische Biochemie, Chirurgische Klinik, Ludwig-Maximilians-Universität München, Nußbaumstr. 20, D-80336 München, Germany
³Adolf-Butenandt-Institut, Ludwig-Maximilians-Universität München, Schillerstr. 42, D-80336 München, Germany
⁴Fakultät für Chemie, Universität Bielefeld, Universitätsstr. 25, D-33615 Bielefeld, Germany
*Corresponding author
e-mail: norbert.schaschke@uni-bielefeld.de

Abstract

The 27-mer peptide CP1B-[1–27] derived from exon 1B of calpastatin stands out among the known inhibitors for \( \mu \)- and m-calpain due to its high potency and selectivity. By systematical truncation, a 20-mer peptide, CP1B-[4–23], was identified as the core sequence required to maintain the affinity/selectivity profile of CP1B-[1–27]. Starting with this peptide, the turn-like region Glu¹(i)-Leu¹(i+1)-Gly¹²(i+2)-Lys¹³(i+3) was investigated. Sequence alignment of subdomains 1B, 2B, 3B and 4B from different mammalians revealed that the amino acid residues in position i+1 and i+2 are almost invariably flanked by oppositely charged residues, pointing towards a turn-like conformation stabilized by salt bridge/H-bond interaction. Accordingly, using different combinations of acidic and basic residues in position i and i+3, a series of conformationally constrained variants of CP1B-[4–23] were synthesized by macrolactamization utilizing the side chain functionalities of these residues. With the combination of Glu(i)/Dab(i+3), the maximum of conformational rigidity without substantial loss in affinity/selectivity was reached. These results clearly demonstrate that the linear peptide chain corresponding to subdomain 1B reverses its direction in the region Glu¹₀-Lys¹³ upon binding to \( \mu \)-calpain, and thereby adopts a loop-like rather than a tight turn conformation at this site.

Keywords: cysteine protease; macrolactamization; peptidomimetic; protease inhibitor.

Introduction

The calpains constitute a family of calcium-dependent cysteine proteases (clan CA, family C2) (Barrett et al., 1998). Among them, the ubiquitous mammalian calpains, m-calpain and \( \mu \)-calpain, are by far the best-characterized members of this family. Both enzymes exhibit their catalytic activity in the cytosol at neutral pH. \( \mu \)-Calpain reaches its half-maximal activity at calcium concentrations in the micromolar range, whereas m-calpain needs millimolar calcium concentrations to be active. The ubiquitous calpains are heterodimeric proteins composed of a large subunit of approximately 80 kDa harboring the catalytic activity and a small 28-kDa subunit proposed to exhibit regulatory activities. Whereas the large subunit is identical for both enzymes, the large subunits share 55–65% sequence homology between the two proteases (for recent reviews see Sorimachi and Suzuki, 2001; Goll et al., 2003). The X-ray structures of calcium-free rat and human m-calpain have elucidated the structural organization of the enzyme (Hosfield et al., 1999; Strobl et al., 2003). In particular, the large subunit is composed of six domains. Two of them (domain Ila and Iib) constitute the catalytic core with an overall papain-like fold.

Besides the local calcium concentration, the endogenous protein-type inhibitor calpastatin plays an important role in the regulation of calpain activity (for a recent review see Wendt et al., 2004). Calpastatin is widely distributed in mammalian tissues. Upon calcium activation, calpastatin specifically binds to the calpains in a reversible fashion. Calpastatin comprises five domains: an N-terminal, so-called L-domain without inhibitory activity and four repetitive inhibitory domains (1–4), each of them organized in three subdomains (A, B and C). Only subdomain B is able to block the catalytic activity of calpain, whereas peptides representing subdomains A and C activate calpain in vitro (Tompa et al., 2002) and in the cell (Bánoczci et al., 2007). Subdomains A and C have been shown to bind to domain IV of the large and domain VI of the small subunit of calpain, respectively (Takano et al., 1995), subdomain B is proposed to interact with an area near the active site (domain Ila and/or Iib).

In the last decade, several attempts have been undertaken to obtain a detailed picture of the interaction mode of calpastatin with calpain at the atomic level. Of particular interest in this context is the bound conformation of the 27-mer peptide, CP1B-[1–27] (compound 1; see Figure 1A), derived from subdomain 1B of human calpastatin, because this peptide inhibits calpain almost as efficiently as the parent whole inhibitory domain 1 of the protein (Maki et al., 1989). Amongst the synthetic calpain inhibitors that have been developed so far (for reviews see Wells and Bihovsky, 1998; Donkor, 2000), CP1B-[1–27] (compound 1) is well suited as a tool to study the (patho)physiological roles of the ubiquitous calpains (Gil-Parrado et al., 2003). For now, investigations of domain 1 of human (Uemori et al., 1990) and porcine
2003). Additionally, the residues Thr17, Ile18 and Pro19 have combination of a b-helix or a turn-like conformation (Ishima et al., 1991). The crucial residues Leu11 and Gly12 (Betts et al., 2003), occupying the positions i+1 and i+2 of the putative turn, respectively, are flanked sequentially by an acidic and a basic amino acid residue that possibly stabilize the turn-like conformation via salt bridge formation and provide the chemical prerequisite for macrolactamization. In this study, we investigated the influence of macrolactamization at this site on the inhibitory potency of CP1B in a systematical manner. To reduce the synthetic effort, a truncated version of CP1B, the 20-mer CP1B-[4–23] (7), was used. We identified CP1B-[4–23] (7) as core sequence necessary to essentially maintain the affinity/selectivity profile of full-length CP1B, taking into account the selectivity aspect that had been neglected in the previously published truncation studies (Betts and Anagil, 2004).

**Results**

Selectivity profiles of truncated linear calpastatin peptides

To assess the selectivity profile of truncated calpastatin peptides within the group of papain-like cysteine proteases, we have selected cathepsin B and L as representative probes. Three groups of truncated calpastatin peptides were synthesized on solid support by the standard Fmoc/tBu scheme. The first and the second group comprise N-terminally and C-terminally truncated variants of CP1B-[1–27] (1), respectively, whereas the third group includes peptides truncated at both termini (Figure 1A). The measured Ki values of these peptides for the inhibition of μ-calpain, cathepsin B and L are summarized in Table 1. N-terminal truncation by three and five (3 and 4, respectively) as well as C-terminal truncation by two and four amino acid residues (5 and 6, respectively) does not influence the affinity/selectivity profile significantly compared to 1. Among the calpastatin peptides truncated at both termini, CP1B-[4–23] (7) is the most potent inhibitor (K_i = 26 nM), which blocks the proteolytic activity μ-calpain by a factor of 1000 more efficiently than that of cathepsin L. Moreover, peptide 7 marks a limit for the truncation tolerated at both termini. Beyond this limit, as clearly documented by 8 and 9, a total loss of affinity and selectivity was observed. To reduce the synthetic effort, we have replaced CP1B-[1–27] (1) by CP1B-[4–23] (7) in all further SAR-investigations.

SAR studies based on linear calpastatin peptides

To gain information on the individual roles of the two turn-like structural elements, the inhibitory properties of the

---

**Figure 1** Structure of linear (A) and conformationally constrained (B) calpastatin peptides.

Peptide 12: Lys⁷ represents D-amino-3,6-dioxoacetic acid replacing the tripeptide portion Arg¹⁴-Glu¹⁵-Val¹⁶ spatially; peptide 13: the lower case letters indicate the corresponding D-amino-acids.
peptides 10 and 11, representing the first and second half of CP1B-[1–27] (1), respectively, were analyzed (Table 1). In particular, peptide 10 comprising the turn-like sequence Glu^{10}-Leu^{11}-Gly^{12}-Lys^{13} is only a poor inhibitor of \( \mu \)-calpain (\( K_i \)=6.9 \( \mu \)M). Similarly, peptide 11 that includes the residues Pro^{10}-Lys^{11}-Tyr^{12}-Arg^{13} being part of the second turn-like structural element, inhibits \( \mu \)-calpain with even lower affinity (\( K_i \)=35 \( \mu \)M). Considering the tripeptide sequence Arg^{12}-Glu^{13}-Val^{14} merely as a linker that connects the two turn-like structural elements, a peptide was designed where this connecting sequence was replaced by a non-peptidic spacer. The flexible PEG amino acid 8-amino-3,6-dioxoacetoic acid was selected as a spacer, which should provide a spatial substitution for the tripeptide portion. The kinetic analysis of this peptide 12 revealed poor inhibitory properties (\( K_i \)=11.7 \( \mu \)M). Furthermore, we applied the retro-inverse approach on the sequence of our truncated calpastatin peptide CP1B-[4–23] (7). It turned out that the corresponding retro-inverse derivative 13 is almost completely inactive (\( K_i \)=726 \( \mu \)M).

**Sequence alignment of the turn-like region Glu^{10}-Leu^{11}-Gly^{12}-Lys^{13} of CP1B-[4–23] (7)**

In a next step, to obtain a more precise picture of the conformation that the core sequence (7, CP1B-[4–23]) adopts upon binding to \( \mu \)-calpain, the turn-like region, i.e., Glu^{10}-Leu^{11}-Gly^{12}-Lys^{13} was investigated. A systematic amino acid sequence alignment of subdomains 1B, 2B, 3B and 4B of calpastatin revealed that this region is highly conserved among different species (Figure 2). In particular, the residues i-1 (Leu/Cys) and i+2 (Gly) of the turn-like region are flanked by a basic and an acidic residue in a pairwise manner. In the case of subdomain 1B, position i is occupied by a Glu and position i+3 by a Lys residue, whereas in the case of subdomains 2B, 3B and 4B the situation is reversed (Lys in position i and Glu in position i+3). Only subdomain 1B from rat and mouse, as well as bovine subdomain 3B, differ from this pattern. Furthermore, the position preceding this turn-like region (i-1) is occupied by an acidic amino acid residue (Asp/Glu), the position following this turn-like region (i+4) by a basic amino acid residue (Arg/Lys). However, amongst all species included in the alignment, the subdomains 2B deviate substantially from this pattern.

**Table 1 Activity/selectivity profiles of linear and conformationally constrained calpastatin peptides.**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>( \mu )-Calpain ( K_i (\mu \text{M})^a )</th>
<th>Cathepsin L ( K_i (\mu \text{M})^a )</th>
<th>Cathepsin B ( K_i (\mu \text{M})^a )</th>
<th>Ratio, CL/( \mu )-calpain</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.0002^a</td>
<td>6^b</td>
<td>( \geq 500^p )</td>
<td>30 000</td>
</tr>
<tr>
<td>2</td>
<td>6^a</td>
<td>6^p</td>
<td>( \geq 500^p )</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>0.0006</td>
<td>9.5</td>
<td>( \geq 600 )</td>
<td>15 830</td>
</tr>
<tr>
<td>4</td>
<td>0.0022</td>
<td>9.7</td>
<td>( \geq 600 )</td>
<td>4410</td>
</tr>
<tr>
<td>5</td>
<td>0.0005</td>
<td>45</td>
<td>( \geq 600 )</td>
<td>90 000</td>
</tr>
<tr>
<td>6</td>
<td>0.0008</td>
<td>21</td>
<td>( \geq 600 )</td>
<td>26 250</td>
</tr>
<tr>
<td>7</td>
<td>0.026</td>
<td>20</td>
<td>n.d.</td>
<td>1000</td>
</tr>
<tr>
<td>8</td>
<td>0.093</td>
<td>15</td>
<td>4.3</td>
<td>161</td>
</tr>
<tr>
<td>9</td>
<td>17</td>
<td>124</td>
<td>( \geq 900 )</td>
<td>7.3</td>
</tr>
<tr>
<td>10</td>
<td>6.9</td>
<td>1.0</td>
<td>60</td>
<td>0.14</td>
</tr>
<tr>
<td>11</td>
<td>35</td>
<td>10</td>
<td>( \geq 200 )</td>
<td>0.29</td>
</tr>
<tr>
<td>12</td>
<td>11.7</td>
<td>28</td>
<td>n.d.</td>
<td>2.4</td>
</tr>
<tr>
<td>13</td>
<td>726</td>
<td>44</td>
<td>32</td>
<td>0.06</td>
</tr>
<tr>
<td>14</td>
<td>0.036</td>
<td>15</td>
<td>( \geq 400 )</td>
<td>420</td>
</tr>
<tr>
<td>15</td>
<td>0.039</td>
<td>13</td>
<td>( \geq 400 )</td>
<td>330</td>
</tr>
<tr>
<td>16</td>
<td>0.027</td>
<td>18</td>
<td>( \geq 400 )</td>
<td>230</td>
</tr>
<tr>
<td>17</td>
<td>1.4</td>
<td>1.3</td>
<td>3.7</td>
<td>0.9</td>
</tr>
</tbody>
</table>

\( ^a \)Mean of 5–10 experiments with different inhibitor concentrations (SD \( <10\% \)). Values marked by ‘\( ^b \)’ are 10-fold the highest used inhibitor concentration that resulted in less than 10% inhibition.

\( ^p \)Data taken from Gil-Parrado et al. (2003). n.d., not determined.

**Design and synthesis of conformationally constrained versions of CP1B-[4–23] (7)**

The consensus pattern Glu/Lys^{10} (i) – Leu/Cys^{11} (i+1) – Gly^{12} (i+2) – Lys/Glu^{13} (i+3) extracted from the sequence alignment suggests that a loop-like structure could be stabilized by a salt bridge/H-bond interaction of the pairs of amino acids with oppositely charged side chains in position i and i+3. To test this hypothesis, the side chains of Glu^{10} and Lys^{13} within CP1B-[4–23] (7) were covalently linked by macroactamization. Furthermore, using different combinations of acidic and basic amino acid residues in positions i and i+3, the conformational freedom was restricted successively via the side chain functions of these residues in a systematic manner. Thereby, the sequence is forced into a restricted conformation at this position, starting from a loose loop-like structure and ending up in a tight turn. The sequences of the four constrained peptides are shown in Figure 1B. The synthesis of these conformationally constrained variants of CP1B-[4–23] (7) is outlined in Figure 3. The peptides were synthesized on solid support by the standard Fmoc/tBu scheme. The basic residues in position i+3 were incorporated into the peptide chain Aloc-protected and the acidic residues in position i Al-protected. Prior
Figure 2 Amino acid sequence alignment of subdomains 1B, 2B, 3B and 4B of calpastatin from different mammalian species. Positions of shown residues within the complete sequence are given in parentheses. The amino acid residues reflecting the consensus sequence pattern of the turn-like region (residues 10–13 which are supposed to constitute the turn as well as the preceding residue 9 and the following residue 14) are highlighted (residues i-1/iq4: black; residues i/iq3: dark-gray; residues iq1/iq2: light-gray).

Figure 3 Synthesis of the conformationally constrained calpastatin peptides.
Reaction conditions: (i) a. piperidine/NMP (1:5, v/v), b. Fmoc-Xaa-OH/HBTU/HOBt/DIEA (1:1:1:2, 10 equiv.), NMP, 10 cycles of automated single coupling; (ii) a. piperidine/NMP (1:5, v/v), b. Fmoc-Xaa-OH/HBTU/HOBt/DIEA (1:1:1:1, 4 equiv.), DMF, 4 cycles of manual double coupling; (iii) Pd(PPh3)4 (0.1 equiv.), PhSiH3 (5 equiv.), DCM; (iv) PyBOP/HOBt/DIEA (1:1:2, 5 equiv.), DMF; (v) a. piperidine/NMP (1:5, v/v), b. Fmoc-Xaa-OH/HBTU/HOBt/DIEA (1:1:1:2, 10 equiv.), NMP, 6 cycles of automated double coupling; (vi) a. Fmoc-Xaa-OH/NMP (1:5, v/v), b. Ac2O/2,6-lutidine/DMF (5:6:89, v/v/v); (vii) TFA/H2O/TIS (95:2.5:2.5, v/v/v).

Inhibition of μ-calpain by conformationally constrained variants of CP1B-[4–23] (7)
A comparison of the Ki values of the linear core sequence 7 with its cyclic counterpart 14 clearly shows that macro lactamization via the side chains of Glu10 and Lys13 does not affect the inhibitory profile (Ki of 0.026 μM vs. 0.036 μM, respectively). Furthermore, starting from the amino acid pair Glu(i)/Lys(i+3) (14), over Glu(i)/Orn(i+3) (15), and Glu(i)/Dab(i+3) (16), to Asp(i)/Dab(i+3) (17), the size of the macrolactam ring decreases stepwise by one CH2-group. The Ki values of these peptides are summarized in Table 1. The peptides 14, 15 and 16 are as potent and selective as the linear core sequence 7. However, the constraint introduced by the amino acid pair Asp(i)/Dab(i+3) with the smallest macrolactam ring is accompanied by a substantial loss in affinity in comparison to 7 (Ki of 1.4 μM vs. 0.026 μM, respectively).

3D visualization of the conformationally constrained region
A 3D model of the conformationally constrained part of 16 induced by the macrolactam ring was built (Figure 4). Besides the modified consensus sequence portion (i–(i+3), (Glu10-Leu11-Gly12-Dab13)), the observation was taken into account that within the subdomains 1, 3 and 4 the positions (i–1) preceding and (i+4) following this...
region are occupied in a systematical manner by an acidic and a basic amino acid residue, respectively (see above). Therefore, the residues i-1 (Glu) and i+4 (Arg) were also included in the model. Inspection of the model revealed a spatial array of a positively charged, a hydrophobic, and a negatively charged residue presented by the loop region.

Discussion

To reduce the synthetic effort for SAR investigations with the full-length calpastatin peptide, CP1B-[1–27] (1), we were interested in a core sequence that reliably reproduces the affinity/selectivity profile of 1. Extending earlier rough determinations of the affinity of truncated calpastatin peptides (Uemori et al., 1990), the group of Anagli has investigated the influence of truncation on the affinity of 1 (Betts and Anagli, 2004). Including the aspect of selectivity, we have reexamined these experiments, using, besides cathepsin B, in particular cathepsin L as a reliable marker to estimate the selectivity of inhibition within the family of papain-like cysteine proteases. Generally, our data are consistent with the affinities previously reported (Betts and Anagli, 2004). N-terminal truncation of the full-length sequence by three amino acids (3, \(K_i=0.0006\) \(\mu\)M; ratio CL/\(\mu\)-calpain 15 830) as well as C-terminal truncation by four residues (6, \(K_i=0.0008\) \(\mu\)M; ratio CL/\(\mu\)-calpain 26 250) does not influence the inhibition profile compared to 1 (\(K_i=0.0002\) \(\mu\)M; ratio CL/\(\mu\)-calpain 30 000). A combination of both truncations yielded a calpastatin peptide of 20 amino acids length, i.e., CP1B-[4–23] (7), which still displayed a reasonable inhibitory profile (\(K_i=0.026\) \(\mu\)M; ratio CL/\(\mu\)-calpain 1000). However, beyond this level of truncation (compounds 8 and 9) a drastic loss of affinity and selectivity was observed. Thus, 7 represent a core sequence comprising the minimum requirements to selectively block the proteolytic activity of \(\mu\)-calpain among other papain-like cysteine proteases.

NMR investigations of 1 in DMSO (Ishima et al., 1991) suggest that the sequence portions Glu11-Leu11-Gly12-Tyr12-Pro13-Lys13 and Pro20-Lys21-Tyr22-Arg23 adopt turn-like conformations. Furthermore, by a systematical \(\beta\)-Ala scan the residues Leu11 and Gly12, as well as the residues Thr17, Ile18, and Pro19, have been identified to be crucial for the affinity of 1 (Betts et al., 2003). Together, these observations point towards two hot spots (Betts and Anagli, 2004) responsible for binding of 1 to calpain. Surprisingly, peptides 10 and 11 comprising the individual first and second hot spot region, respectively, exhibit only poor inhibitory activities. Even an equimolar mixture of those peptides did not reconstitute the inhibitory activity of 1 (Betts and Anagli, 2004). Based on these data, Anagli’s group introduced the ‘gate model’ for the inhibition of calpain: the peptide sequence between the two hot spots, i.e., Lys13-Arg14-Glu15-Val16, positioned appropriately by the interactions of the two hot spots with calpain, act as a gate blocking the access of substrates to the active site (Betts and Anagli, 2004). To gain more information on the role of this gate, we have designed peptide 12, based on the core sequence 7, where the amino acid residues Arg14-Glu15-Val16 are replaced by the flexible PEG amino acid 8-amino-3,6-dioxaoctanoic acid that should provide a correct spatial arrangement of the two hot spot regions. However, the low affinity of 12 (\(K_i=11.7\) \(\mu\)M) supports the notion that not only the correct spacing of the crucial binding elements is required to restore the affinity of 1, but also the correct conformation provided by the tripeptide sequence. Furthermore, the retro-inverso peptide of the core sequence (i.e., 13, \(K_i=726\) \(\mu\)M) demonstrates that besides the correct spatial positioning of the amino acid side chains, the peptide backbone also has a strong influence on the affinity.

The fact that the crucial amino acid residues Leu11 and Gly12 (Betts et al., 2003) occupy the positions i+1 and i+2 of the proposed \(\beta\)-turn (Ishima et al., 1991), respectively, prompted us to analyze the amino acid sequence of this region systematically. The sequence alignment of peptides derived from subdomains 1B, 3B and 4B of different species revealed a consensus sequence for this turn region, i.e., Glu/Asp (i-1) – Lys/Glu (i) – Leu (i+1) – Gly (i+2) – Lys/Glu (i+3) – Arg/Glu (i+4). However, peptides derived from subdomains 2B show a substantially different sequence pattern. Correspondingly, a comparison of the inhibitory potency of the subdomains 1B, 2B, 3B and 4B from rabbit calpastatin has revealed that 2B is by far the least potent inhibitor of \(\mu\)-calpain (Kawasaki et al., 1989). Very recently, these findings have been confirmed by surface plasmon resonance measurements (Hanna et al., 2007). It is striking to observe that in the case of 1B peptides the residues Leu11 and Gly12 are flanked by Glu in position i and Lys in position i+3, whereas in the case of 3B and 4B peptides the basic Lys (position i) is followed by an acidic Glu (position i+3). This points towards a turn conformation at this site that is possibly stabilized by a salt bridge interaction of the oppositely charged side chain functionalities. Furthermore, one would expect that this type of local conformation would orientate the oppositely charged residues in position i-1 and i+4 in a defined spatial manner. To test this hypothesis, 14 was synthesized where the putative salt bridge stabilized turn-like conformation in the parent linear core sequence 7 was covalently fixed by macrocyclization. A comparison of the \(K_i\) values clearly shows that 14 is virtually as potent as 7 (\(K_i=0.036\) \(\mu\)M vs. 0.026 \(\mu\)M). This finding is supportive for a turn-like conformation stabilized by a salt bridge formed between side chain carboxyl function of Glu12 and the side chain amino function of Lys13.

To gain more information about the geometry of this turn-like structure, the local conformational degrees of freedom were successively reduced by stepwise removal of CH2-groups within the macroactam ring. The constraint induced by removal of one CH2-group (15) as well as two CH2-groups (16) does not influence the inhibitory profile substantially compared to 7 (\(K_i=0.039\) \(\mu\)M and 0.027 \(\mu\)M vs. 0.026 \(\mu\)M, respectively). However, removal of three CH2-groups (17), corresponding conformationally to a tight \(\beta\)-turn is accompanied with a severe loss of inhibitory potency (\(K_i=1.4\) \(\mu\)M). These data clearly support the notion that the peptide portion Glu15-Leu11-Gly12-Lys13 adopts a more loop-like structure rather than a
β-turn as earlier proposed (Ishima et al., 1991), pointing to a chain reversal of the 1B peptide at this site in the peptide sequence. Moreover, it seems that in contrast to the constrained variants 14, 15 and 16, in peptide 17 the amino acid portion CP1B-[4–9] preceding the turn region as well as the amino acid portion CP1B-[14–23] following the turn region cannot adopt the proper orientation and/or conformation upon binding to μ-calpain.

Based on X-ray data of the interaction of a subdomain 1C peptide with domain VI of calpain and the homology model of the interaction of a subdomain 1A peptide with domain IV of calpain, models for the interaction of an entire inhibitory domain of calpastatin with calpain have been proposed (Todd et al., 2003; Wendt et al., 2004). To properly connect the subdomains 1A and 1C, both models claim a reversal of the peptide chain within the region corresponding to subdomain 1B. Our data derived from the conformationally constrained variants of 7 presumably indicate that the chain reversal is centered at the tetrapeptide portion Glu10-Leu11-Gly12-Lys13.

The consensus sequence of the loop site revealed that the polarity pattern of the residues i-1 (Glu or Asp: acidic) preceding and i+4 (Lys, Arg: basic) following this loop is highly conserved (Figure 2). The consequence of the loop-like structure is that these particular residues occupy spatially defined positions. In combination with the hydrophobic side chain of Leu11, which has been shown to be highly sensitive to modifications (Bettis and Anagli, 2004), these residues could be responsible for the correct positioning of the 1B peptide on the calpain surface by addressing complementary binding pockets, and thereby blocking the access of substrates to the active site.

In conclusion, based on conformationally restricted variants of subdomain 1B of human calpastatin, a first model of the biologically active conformation has been provided. In particular, macro lactamization studies revealed that the tetrapeptide portion Glu11-Leu12-Gly13-Lys14 adopts a loop-like conformation inducing a reversal of the peptide chain at this site upon binding to μ-calpain.

Materials and methods

Materials

Solvents and reagents were of the highest purity commercially available and were used without further purification. Amino acid derivatives were purchased from Iris Biotech GmbH (Marktredwitz, Germany) and Fluka (Buchs, Switzerland), the Rink-Amide resin from NovaBiochem/Merck Biosciences AG (Läufelfingen, Germany), Fmoc-8-amino-3,6-dioxaoctanoic acid from NeoMPS SA (Strasbourg, France), CP1B-[1–27] (7) as well as its scrambled derivative 2 from Calbiochem (Bad Soden, Germany). Automated peptide synthesis was carried out on a batch synthesizer 433A (Applied Biosystems, Foster City, CA, USA) equipped with a UV/vis detector PE series 200 from Perkin Elmer (Überlingen, Germany) for monitoring of the Fmoc-cleavage. Manual peptide synthesis as well as all other manually performed synthetic steps were carried out on an IKA KS 130 basic laboratory shaker (IKA Werke GmbH, Staufen, Germany) using plastic syringes type Discard II from Becton Dickinson (Franklin Lakes, NJ, USA) equipped with PE frits (35 μm pores size) and PE stoppers from Roland Vetter Laborbedarf (Ammerbuch, Germany). LC-MS mass spectra were recorded with a PE Scieix API 165 ESI-MS spectrometer and a microgradient system 140 C (Applied Biosystems, Framingham, MA, USA). Nucleosil 100-5 C8 RP columns (Macherey-Nagel, Düren, Germany) were used with a linear gradient of 0.1% TFA in H2O and 0.08% TFA in CH3CN from 95:5 to 5:95. The MS spectra were processed with PE Sciex BioMultiView software (V.1.3.1, Applied Biosystems). Analytical RP-HPLC was performed with Waters equipments using Chromolith Performance RP-18e (100×4.6 mm; WWR Internationale GmbH, Darmstadt, Germany) columns and eluting with linear gradients from 5% CH3CN in H2O/0.95% H3PO4 (2% in H2O) to 90% CH3CN in H2O/10% H3PO4 (2% in H2O) within 6 min at a flow rate of 3 ml/min. For preparative reversed-phase HPLC, Gilson-Abled equipment (Middleton, WI, USA) and Nucleosil 100-5 C18 columns (Macherey-Nagel) were used and compounds were eluted with linear gradients of 0.1% TFA in H2O and 0.08% TFA in CH3CN. The 3D model of the conformationally constrained region of 16 was built using the program HyperChem™ (Release 3, Hypercube Inc., Gainesville, FL, USA).

Syntnesis of linear calpastatin peptides

The linear peptides were synthesized using an automated peptide synthesizer according to the Fmoc/Ibu strategy. Fmoc-protected amino acid with the following side-chain protecting groups were used: OtBu (Asp and Glu); tBu (Ser, Thr and Tyr), Boc (Lys) and Pbf (Arg). In the case of the retro-inverso peptide 13, the corresponding D-amino acids were used. The coupling was performed with Fmoc-Xaa-OH/HBTU/HOBt/DIEA (1:1:1:2, 10 equiv.) in NMP using the standard FastMoc™ protocol (Applied Biosystems) for single and double coupling, respectively. Unless otherwise stated, single coupling was performed. The loading of the Rink-Amid resin (0.1 mmol) with the first amino acid was also performed using the synthesizer under the same conditions as for a single coupling step. After each coupling step, the Fmoc group was cleaved using piperidine/NMP (1:5, v/v). The acetylation of the N-terminal amino group was performed manually using Ac2O/2,6-lutidine/DMF (5:6:89, v/v/v). The final deprotection was also carried out manually using TFA/H2O/TIS (95:2.5:2.5, v/v/v). The crude peptides were precipitated by adding the cleavage solution dropwise to ice-cold tert-butyl methyl ether/hexane (2:1, v/v). The precipitate was collected by centrifugation and purified by preparative RP-HPLC. Homogeneous fractions were pooled and lyophilized.

\[
\text{Ac-SSTIEELKREVTPPYYRELLA-NH}_2 \quad (3) \quad \text{Double coupling starting with the 4th Glu; colorless lyophilisate: yield: 164 mg (58%); HPLC (tR=2.1 min); ESI-MS: m/z=1418.0 [M+2H]^2+, 945.8 [M+3H]^3+, 709.4 [M+4H]^4+; M+2834.29 calculated for } C_{131}H_{199}N_{31}O_{35}.
\]

\[
\text{Ac-TYIEELKREVTPPYYRELLA-NH}_2 \quad (4) \quad \text{Double coupling starting with the 4th Glu; colorless lyophilisate: yield: 183 mg (69%); HPLC (tR=2.1 min); ESI-MS: m/z=1330.8 [M+2H]^2+, 887.4 [M+3H]^3+, 666.0 [M+4H]^4+; M=2660.13 calculated for } C_{131}H_{199}N_{31}O_{35}.
\]

\[
\text{Ac-PDMSTYIEELKREVTPPYYRELLA-NH}_2 \quad (5) \quad \text{Double coupling starting with the 4th Glu; colorless lyophilisate: yield: 197 mg (66%); HPLC (tR=2.0 min); ESI-MS: m/z=1498.0 [M+2H]^2+, 998.4 [M+3H]^3+, 748.8 [M+4H]^4+; M=2993.39 calculated for } C_{142}H_{210}N_{32}O_{40}S.
\]

\[
\text{Ac-PDMSTYIEELKREVTPPYYRELLA-NH}_2 \quad (6) \quad \text{Double coupling starting with the 3rd Glu; colorless lyophilisate: yield: 195 mg (71%); HPLC (tR=1.9 min); ESI-MS: m/z=1376.4 [M+2H]^2+,
\]
Inhibition of μ-calpain by calpastatin peptides

89

918.0 [M+3H]+, 688.6 [M+4H]+; M–2751.18 calculated for C_{15}H_{30}N_{22}O_{21}S.

Ac-STYIEHLGKREVTPPKYR-NH_{2} (7) Double coupling starting with the 3rd Glu; colorless lyophilisate: yield: 180 mg (75%); HPLC (t_{R}=1.8 min); ESI-MS: m/z=1044.8 [M+2H]+, 803.4 [M+3H]+, 603.0 [M+4H]+; M–2407.77 calculated for C_{19}H_{29}N_{20}O_{20}S.

Ac-ASYIEHLGKREVTPPKYR-NH_{2} (8) Double coupling starting with the 3rd Glu; colorless lyophilisate: yield: 148 mg (66%); HPLC (t_{R}=1.8 min); ESI-MS: m/z=1117.6 [M+2H]+, 745.4 [M+3H]+, 559.4 [M+4H]+; M–2233.62 calculated for C_{19}H_{29}N_{20}O_{20}S.

Ac-STYIEHLGKREVTPPK-NH_{2} (9) Double coupling starting with the 3rd Glu; colorless lyophilisate: yield: 153 mg (73%); HPLC (t_{R}=1.8 min); ESI-MS: m/z=1044.8 [M+2H]+, 803.4 [M+3H]+, 603.0 [M+4H]+; M–2407.77 calculated for C_{19}H_{29}N_{20}O_{20}S.

Ac-DPMSTFYIEELG-NH_{2} (10) Colorless lyophilisate: yield: 109 mg (72%); HPLC (t_{R}=2.0 min); ESI-MS: m/z=1511.8 (M+H)+, 756.2 [M+2H]+; M–1510.76 calculated for C_{19}H_{29}N_{20}O_{20}S.

Ac-REVTPPKYRELLA-NH_{2} (11) Colorless lyophilisate: yield: 134 mg (78%); HPLC (t_{R}=1.8 min); ESI-MS: m/z=1727.4 [M+2H]+, 864.0 [M+3H]+, 576.2 [M+4H]+; M–1726.16 calculated for C_{19}H_{29}N_{20}O_{20}S.

Ac-STYIEHLGK-(CH_{2}O)_{2}-CH_{2}C(O)-TIPPKYR-NH_{2} (12) Double coupling starting with the 2nd Glu; colorless lyophilisate: yield: 95 mg (44%); HPLC (t_{R}=1.8 min); ESI-MS: m/z=1084.8 [M+2H]+, 723.8 [M+3H]+; M–2168.45 calculated for C_{19}H_{29}N_{20}O_{20}S.

Ac-rykptiverkleeytts-NH_{2} (13) Double coupling starting with Val; colorless lyophilisate: yield: 98 mg (41%); HPLC (t_{R}=2.3 min); ESI-MS: m/z=1206.0 [M+2H]+, 803.6 [M+3H]+, 603.0 [M+4H]+; M–2407.8 calculated for C_{19}H_{29}N_{20}O_{20}S.

Synthesis of conformationally constrained calpastatin peptides

The peptide sequence REVTPPKYR was synthesized using a Rink-Amide resin (0.1 mmol), as described for the linear peptides. After each coupling step, the resin was washed with DMF, DCM and iPrOH sequentially (3×3 ml each). To cleave the allyl-based protecting groups, the N-terminally Fmoc-protected resin-bound peptide was treated (3×15 min) with Pd(PPh3)4 (0.1 equiv.) and PhSiH3 (5 equiv.) in dry DCM (3 ml) under an argon atmosphere. Upon each treatment, the resin was washed with dry DCM (2×3 ml). Finally, the resin was washed with DMF, DCM and iPrOH sequentially (3×3 ml each). The macroactamization (2×120 min coupling time) was carried out with PyBOP/HOBt/DIEA (1:1:2, 5 equiv.). In the reaction mixture of 17 (X=N-Bz, X=Asp, X=Glu), it was necessary to repeat the macroactamization step a third time overnight to complete the reaction. The remaining sequence was built up using the synthesizer (double coupling, 10 equiv.), N-terminal acetylation, final deprotection and purification was performed as described for the linear peptides.

Ac-rykptiverkleeytts-NH_{2} (13) Double coupling starting with Val; colorless lyophilisate: yield: 98 mg (41%); HPLC (t_{R}=2.3 min); ESI-MS: m/z=1206.0 [M+2H]+, 803.6 [M+3H]+, 603.0 [M+4H]+; M–2407.8 calculated for C_{19}H_{29}N_{20}O_{20}S.

Inhibition assays and Kd determination

Enzymes and substrates were obtained as outlined in Gil-Parrado et al. (2003). Inhibition of μ-calpain (0.5–1 nm) was measured at 12°C (to slow down inactivation by autolysis) with the fluorogenic substrate Suc-LY-amc (400 μM) in 50 mM Tris/HCl, pH 7.5, 100 mM NaCl, 0.015% Brij-35, (calpain buffer) and 150–200 μM CaCl2, 1 mM diithiothreitol (DTT). Cathepsin L (5 pmol) was assayed at 25°C with Z-FR-amc (4 μM) and cathepsin B (25 pmol) at 30°C with Z-FR-amc (10 μM) in 0.25 mM sodium acetate, pH 5.5, 2 mM EDTA, 0.015% Brij-35. 1 mM diithiothreitol, freshly added. Continuous assays were performed recording fluorescence (excitation 380 nm, emission 460 nm) essentially as described in Machleidt et al. (1993). The enzymes were preactivated with DTT in the presence of substrate and CaCl2 (only with μ-calpain). Then approximately 5–10 different inhibitor concentrations were added in maximal 1% of the total test volume of 500 μl (dissolved and prediluted in calpain buffer) and the reaction was followed until equilibrium was reached. Kd values for the inhibition of μ-calpain were obtained from presteady state kinetics, fitting the progress data by non-linear regression analysis to the integrated equation of Morrison (1982). From the rate constants k_{cat} and k_{on}, the Kd values were calculated as Kd=k_{cat}/k_{on}. For cathepsin L B, the inhibition constants were calculated from the initial and steady state rates using the equation of classical inhibition. Kd values were corrected for competition with the substrates. In cases of less than 10% inhibition, the Kd value was assumed to be at least 10-fold the highest used inhibitor concentration.

Acknowledgments

The study was supported by the DFG with a grant (SCHA 1012/1-2).

References


References


