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Folding transitions in calpain activator peptides studied by solution NMR spectroscopy

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Calpastatin, the endogenous inhibitor of calpain, a cysteine protease in eukaryotic cells, is an intrinsically unstructured protein, which upon binding to the enzyme goes through a conformational change. Peptides calpA (SGKSGMDAALDDLIDTLGG) and calpC (SKPIGPDDAIDALSSDFTS), corresponding to the two conserved subdomains of calpastatin, are known to activate calpain and increase the Ca^{2+} sensitivity of the enzyme. Using solution NMR spectroscopy, here we show that calpA and calpC are disordered in water but assume an α-helical conformation in 50% CD_3OH. The position and length of the helices are in agreement with those described in the literature for the bound state of the corresponding segments of calpastatin suggesting that the latter might be structurally primed for the interaction with its target. According to our data, the presence of Ca^{2+} induces a backbone rearrangement in the peptides, an effect that may contribute to setting the fine conformational balance required for the interaction of the peptides with calpain. Copyright © 2009 European Peptide Society and John Wiley & Sons, Ltd.

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Keywords: calpain; calpastatin; calcium-binding; inhibitor; conformational change; folding; intrinsically unstructured proteins; order–disorder transition; NMR spectroscopy

Introduction

Calpains are intracellular cysteine proteases whose action leads to the limited proteolysis of a variety of substrate proteins in eukaryotes [1,2]. These proteins in turn are involved in diverse cellular processes such as cell adhesion and motility [3], signal transduction [4], cell-cycle progression [5], cell death [6], exocytosis [7], and regulation of gene expression [8]. The misregulation of calpain activity has long been associated with acute neurological disorders (e.g. stroke) [9] and more recently with Alzheimer disease [10], and a number of other pathological conditions, such as type 2 diabetes, gastric cancer, and muscular dystrophy [11].

The activation of calpain is controlled by the free intracellular Ca^{2+} concentration and by the action of calpastatin, the endogenous inhibitor of the enzyme [12]. Calpastatin is a 77 kDa IUP [13] with four equivalent inhibitory domains, each capable of binding a calpain molecule and each containing three conserved subdomains, A, B, and C [14]. Among the three subdomains, it is subdomain B that inhibits calpain [15], whereas subdomains A and C increase the overall affinity of the interaction. X-ray diffraction studies have shown that the latter two fold as amphipathic alpha-helices upon binding to the Ca^{2+}-induced hydrophobic cleft of the enzyme, whereas region B binds on the two sides of the substrate-binding cleft looping around the active site cysteine to avoid proteolysis [16,17]. It has also been shown by these studies that the fully activated calpain binds ten Ca^{2+} ions and calpastatin recognizes only the Ca^{2+}-induced conformation of the enzyme.

The common theme in the mechanism of action of IUPs such as calpastatin is recognizing and binding to a partner molecule upon which they undergo a conformational change and a portion of them becomes ordered [18–20]. To maintain a sufficiently fast association and dissociation rate in these reactions, a balance between order and disorder is required before binding occurs. To look for signs of this balance in calpastatin we have set out to characterize the conformational transitions in peptides calpA (SGKSGMDAALDDLIDTLGG) and calpC (SKPIGPDDAIDALSSDFTS) corresponding to subdomains A and C of human calpastatin inhibitory domain 1 using solution NMR spectroscopy. Both

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Abbreviations used: *nuclear magnetic resonance, NMR; intrinsically unstructured protein, IUP; dimethyl-formamide, DMF; N,N′-diisopropyl-carbodiimide, DIC; 1-hidroxybenztriazole, HOBt; trifluoroacetic acid, TFA; high pressure liquid chromatography, HPLC; total correlation spectroscopy, TOCSY; rotating frame nuclear Overhauser effect spectroscopy, ROESY; heteronuclear single quantum correlation, HSQC; double pulse field gradient spin echo, DPFGSE; ambiguous restraints for iterative assignment, ARIA; root mean square deviation, RMSD.*

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peptides have been shown to activate human calpain 1 and 2 (also termed μ- and m-calpains) and to increase their sensitivity to Ca^{2+} [21]. Peptide conformations have been investigated in water and in methanol/water mixtures in both the absence and in the presence of Ca^{2+}.

Materials and Methods

Peptide Synthesis and Purification

The acetylated calpA (*Ac*-SGKSGMDAALDDLIDTLGG) and calpC (*Ac*-SKPIGPDDAIDALSSDFTS) peptides were synthesized by solid phase methodology on Rink-amide resin (50 mg, 0.72 mmol/g) using Fmoc chemistry on a Syro (Multisyntech, Written, Germany) peptide synthesizer. The Fmoc protection from the N^{α}-amino groups was removed with 40% piperidine in DMF (2 + 15 min) followed by washing with DMF (6 × 0.5 min). For coupling, amino acid derivatives and DIC and HOBt dissolved in DMF were used in 4 molar excess for the resin capacity. The reaction proceeded for 60 min at room temperature. Then, the resin was washed with DMF (4 × 0.5 min) and the coupling process was repeated. The amino acid side-chain protecting groups were *tert*-butyl ether for Thr, Ser and *tert*-butyl ester for Asp. Side-chain of Lys was protected with *tert*-butyloxycarbonyl group. After the removal of the last N^{α}-Fmoc group acetyl groups were introduced to the *N*-terminus of the peptidyl-resins by 2 equiv acetic anhydride and *N*-DIPEA dissolved in DMF.

After assembly, the peptides were cleaved from the resin by 10 ml TFA using 0.75 g phenol, 0.5 ml distilled water, 0.5 ml thioanisole and 0.25 ml ethanedithiol as scavengers. Crude products were precipitated by dry diethyl-ether, dissolved in 10% acetic acid and freeze-dried. The freeze-dried preparations were purified on FPLC® System (Pharmacia, Uppsala, Sweden). Flow rate was 8 ml/min. Linear gradient elution (0 min 30% B; 60 min 70% B) was generated using 0.1% TFA in water as eluent A and 0.1% TFA in acetonitrile-water (80 : 20, v/v) as eluent B. Peaks were detected at $\lambda = 220$ nm.

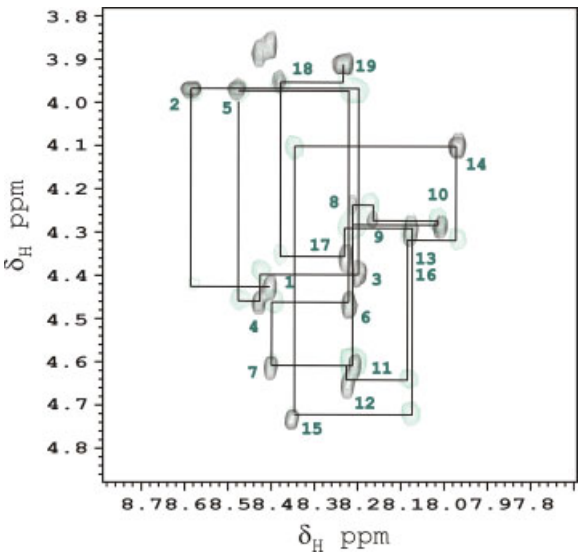

Figure 1. Superimposed fingerprint region from 2D TOCSY (*black*) and 2D ROESY (grey, online green) spectrum of calpA in pure aqueous buffer showing the intra- and interresidue correlations used to help establish sequence-specific resonance assignments. The path over the sequential αH_i-NH_{i+1} connectivities is shown as a continuous *black line*. This figure is available in colour online at www.interscience.wiley.com/journal/jpepsci.

Analytical HPLC was performed on a Knauer (Herbert Knauer GmbH, Berlin, Germany) system using a Phenomenex SYNERGI MAX-RP column (250 × 4.6 mm I.D., 4 µm silica, 80 Å pore size) (Torrance, CA, USA) as a stationary phase. Linear gradient elution, 0 min 0% B; 5 min 0% B; 50 min 90% B, was generated. Flow rate of 1 ml/min was applied at ambient temperature. Peaks were detected at $\lambda = 220$ nm. The samples were dissolved in eluent B. The molecular mass of the peptides was measured by ESI-MS. Positive ion ESI mass spectrometric analysis was performed on a Bruker Esquire 3000 plus (Germany). The samples were dissolved in acetonitrile-water (50 : 50, v/v), containing 0.1% acetic acid.

NMR Spectroscopy and Structure Calculation

One- and two-dimensional NMR experiments were carried out on a *Varian NMR SYSTEM* (600 MHz for ^1H) five-channel spectrometer using a 5-mm indirect detection triple resonance ($^1H^{13}C^{15}N$) z-axis gradient probe. Typically 5–8 mg peptide was dissolved in *either* 600 µl buffer containing 20 mM potassium-phosphate, 50 mM potassium-chloride and 0.05% NaN_3 at pH = 6.5 *or* in 600 µl methanol/distilled water (1 : 1, v/v) mixture. Experiments were performed at 25 °C. In the Ca^{2+} titration experiments, 0.5, 1.0, or 2.0 molar equivalents of $CaCl_2$ were added to the peptide solutions. The ^1H resonances were assigned using the combination of 2D TOCSY, 2D ROESY, and natural abundance ^1H-^{13}C HSQC experiments [22]. Solvent water suppression in the former two experiments was achieved by the DPFGSE technique [23]. The TOCSY experiment implemented a decoupling in the presence of scalar interactions (DIPSI) spin lock for polarization transfer and utilized mixing times (τ_m) up to 120 ms. ROESY experiments were performed at mixing times of 100, 200, 300, and 400 ms. ^1HN-H_α scalar couplings were inferred from 1D ^1H experiments. Interproton distance restraints were obtained from a ROESY experiment performed with $\tau_m = 300$ ms using ARIA, version 2.2) [24,25], a structure calculation program that integrates the assignment of distance restraints with structure refinement. Specifically, ambiguous restraints are assigned during the structure calculation using a combination of unambiguous restraints and an iterative assignment strategy. In each of the seven iterations of the structure calculation, the ten lowest energy structures were used as templates for the next iteration and the seven best structures were used for restraint violation analysis. The computational algorithm in the structure calculation employed torsional angle simulated annealing followed by torsional angle and then Cartesian molecular dynamics cooling stages. Structural refinement was completed in a water shell. The stereochemical quality and structural statistics of the final ensemble were determined using protein structure check (PROCHECK) [26] and PROCHECK NMR [27].

Results and Discussion

Conformation of calpA and calpC in H_2O

The sequential correlations between neighboring backbone amide and alpha protons used to help assign calpA in aqueous buffer are illustrated in a ROESY walk in Figure 1. The full sequence specific ^1H resonance assignments of calpA and calpC under various conditions are given in the Supplementary Information (Table S1–S6). In aqueous buffer the H_α chemical shifts of calpA oscillate around the random coil values suggesting

Table 1. Statistics of the NMR structure and stereochemical quality of peptides calpA and calpC in CD_3OH/H_2O (1 : 1, v/v) at 25 °C

Ensemble RMSD values	calpA	calpC
Average pairwise C_α RMSD, Å	0.67 ± 0.21	1.03 ± 0.30
For the most ordered region (2–16) for calpA and (9–17) for calpC	0.48 ± 0.18	0.63 ± 0.22
Average main-chain RMSD from mean coordinates, Å	0.46 ± 0.20	0.75 ± 0.37
For the most ordered region (2–16) for calpA and (9–17) for calpC	0.31 ± 0.11	0.66 ± 0.24
Statistics		
Ramachandran plot statistics		
Residues in most favored regions [A, B, L], % (No.)	64.3 (9)	78.6 (11)
Residues in additionally allowed regions [a, b, l, p], % (No.)	35.7 (5)	21.4 (3)
Residues in generously allowed regions [~a, ~b, ~l, ~p], % (No.)	0.0 (0)	0.0 (0)
Residues in disallowed regions, % (No.)	0.0 (0)	0.0 (0)
Main-chain statistics		
SD of ω angle, degrees	5.6	3.5
Bad contacts/100 residues	N/A	N/A
C_α chirality, SD of ζ angle, degrees	0.8	1.6
H-bond energy, kcal/mole	0.8	0.9
Overall G-factor	−0.2	−0.1
Side-chain statistics		
χ-1 gauche minus, degrees	N/A	7.4
χ-1 trans, degrees	22.9	12.7
χ-1 gauche plus, degrees	10.3	13.6
χ-1 pooled, degrees	17.4	15.4
χ-2 trans, degrees	17.7	8.2

a disordered conformation. In calpC, on the other hand, particularly in the *C*-terminal half of the chain, the deviations tend to be on the negative side reminiscent of an α-helix. In spite of this, long-range distance restraints are missing from the ROESY spectra of both peptides indicating the lack of sufficient intramolecular contacts to form a stable secondary structure.

Conformation of calpA and calpC in 50% CD_3OH

To mimic the hydrophobic environment that calpA and calpC may encounter upon binding to calpain, the peptides were investigated in CD_3OH/H_2O mixtures. In the presence of 50% methanol, a dramatic shift change occurs in the H_α resonances of calpA (Figure 2A) and the deviations from the random coil values

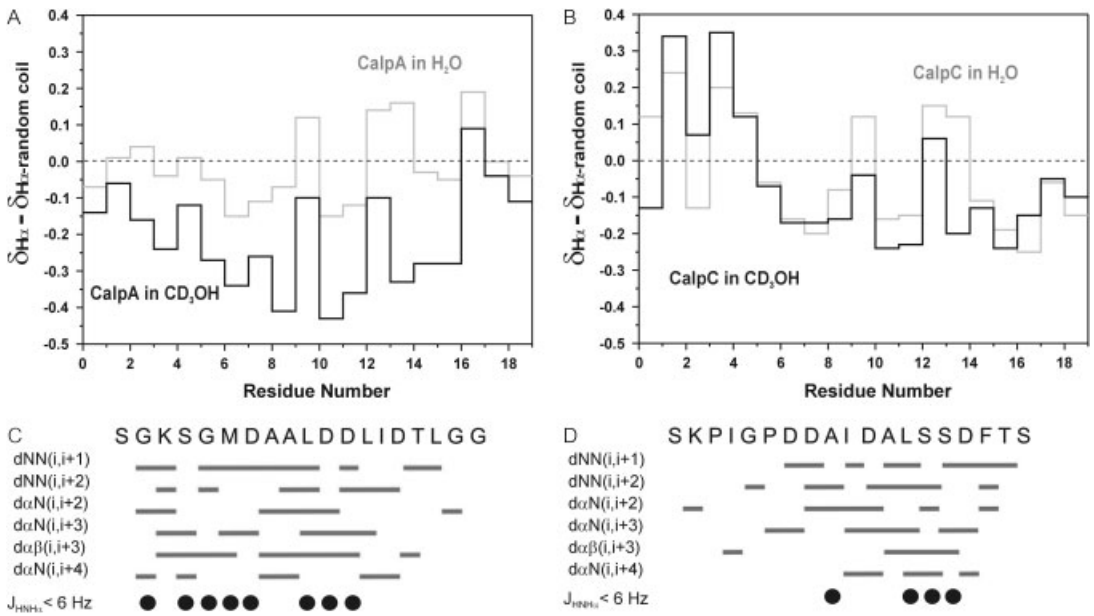

Figure 2. Summary of NMR structural parameters. Deviations of H_α chemical shifts from random coil values for (A) calpA and (B) calpC in pure aqueous buffer (*gray*) and in CD_3OH/H_2O (1 : 1, v/v) (*black*). Diagnostic interatomic distance restraints and HN-H_α scalar couplings observed for (C) calpA and (D) calpC in CD_3OH/H_2O (1 : 1, v/v).

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become more pronounced in calpC as well (Figure 2B). Strong $d_{\alpha N}(i, i+2)$, $d_{\alpha N}(i, i+3)$, $d_{NN}(i, i+2)$, and $d_{\alpha\beta}(i, i+3)$ rotating frame nuclear Overhauser effect (ROE) connectivities as well as $^{3}J_{NH\alpha}$ coupling constants of <6 Hz observed in both peptides are further indications of helix formation. The secondary structure determinants for the two peptides are summarized in Figures 2C and 2D.

All together, among the 349 ROEs we observed for calpA in CD_3OH/H_2O (1 : 1, v/v), 195 were intraresidue, 103 were sequential, and 51 were detected between residues two or more positions apart. In calpC there was a similar distribution of distance restraints. The results of structure calculation for the two peptides are summarized in Figure 3. Independent calculations gave the ten lowest energy structures with a high degree of convergence in both cases (Table 1 and Figures 3A, 3B). Representative structures of the ensembles were determined by calculating the mean coordinates using a subroutine of ARIA. According to this, the chain of calpA adopts an α-helix spanning residues 2–12 followed by a twist in the structure and a three-residue segment of a 3_{10} helix (Figure 3C). The backbone structure of the peptide is well defined with an average RMSD from the mean of 0.224 Å for the backbone atoms in the middle of the chain (residues 4–16) (Figure 3C). Side-chains in the same region have an overall average RMSD from the mean of 0.667 Å. In calpC, due to the two prolines at the beginning of the sequence, the α-helix is shorter and shifted toward the *C*-terminal of the chain spanning residues 9–17 (Figure 3D). We have found no sign of proline *cis-trans* isomerization in the NMR spectra. As reflected by the RMSDs (Figures 3E and 3F) the structure of calpC appears to be somewhat less defined.

Comparison of Peptide Structures in the Free and Bound States

Currently there are three calpain–calpastatin complexes in the PDB database. Todd and coworkers [28] crystallized the calcium-

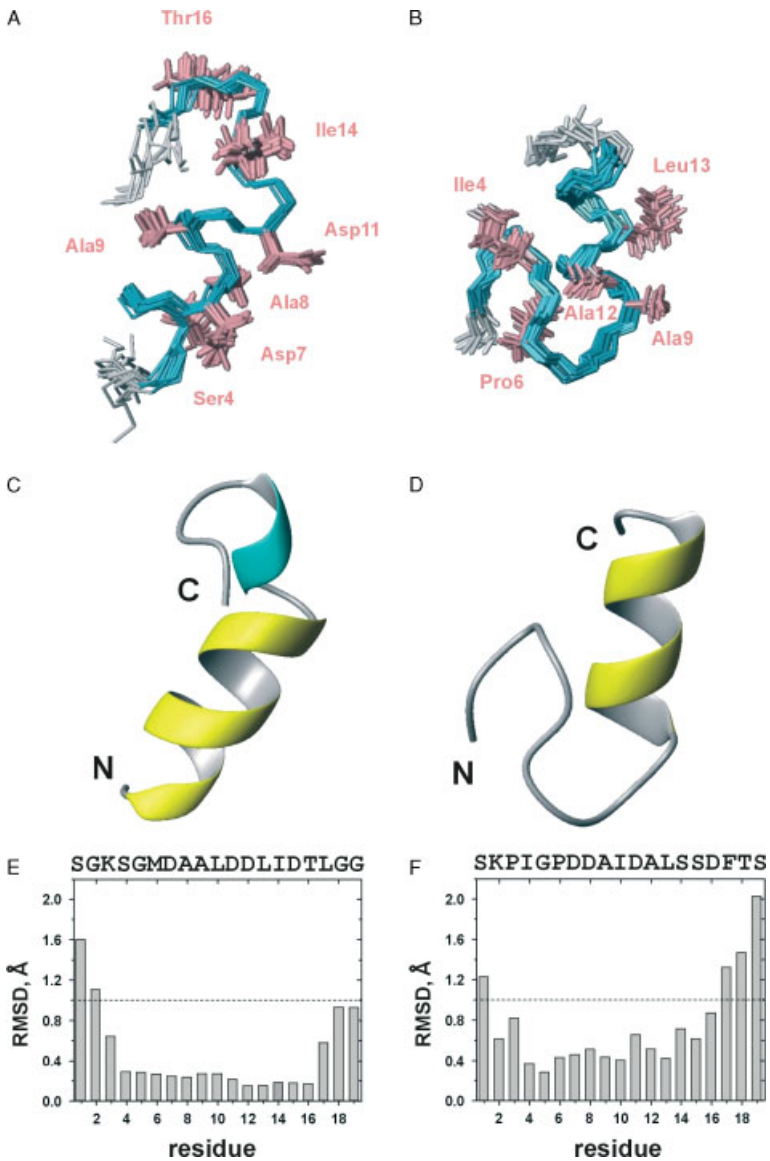

Figure 3. Results of the NMR structure calculation. (A and B) Ensemble of the ten lowest energy conformations of calpA (A) and calpC (B). Backbone for the well-defined section of the chains is in aquamarine. Side-chains with RMSD-s lower than 0.8 are colored in *pink*. (C and D) Ribbon diagram of the mean structure of calpA (C) and calpC (D). α-helices are in *yellow* and the 3_{10} helix is in *cyan*. (E and F) Average backbone RMSD-s from the mean of the ten lowest energy conformations of calpA (E) and calpC (F).

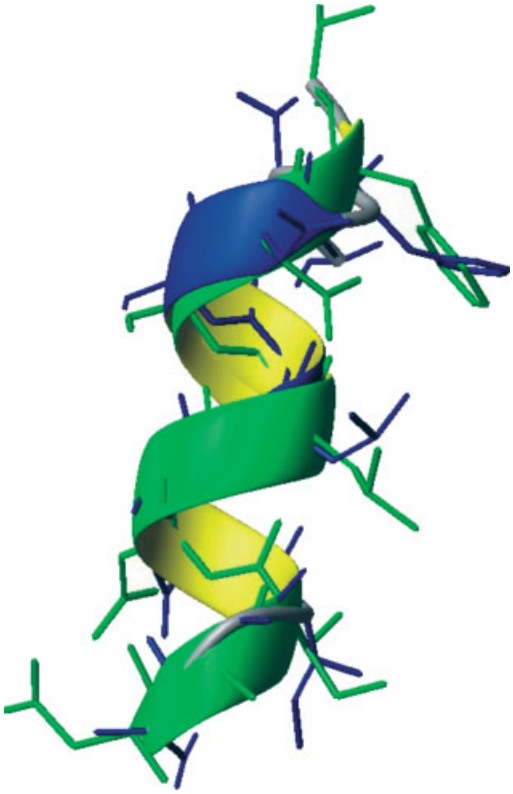

Figure 4. Superposition of the Asp_8–Ser_{19} segment of the representative structure of free calpC in CD_3OH/H_2O (1 : 1, v/v) to the crystal structure of the 12-residue calpastatin-derivative peptide in a complex with domain VI of calpain [28].

binding domain VI of pig calpain with a small molecule inhibitor and a 12-residue peptide corresponding to region Asp_8-Ser_{19} in calpC. According to the X-ray structure of the complex, the bound 12-residue segment of calpC forms an amphipathic α-helix burying its bulky hydrophobic side-chains into calpain. Superposition of the NMR structure we determined for calpC in CD_3OH/H_2O (1 : 1, v/v) and its coordinates in the complex (Figure 4) gives an RMSD of 0.96 Å for the backbone atoms, indicating an overall similarity between the nascent and bound forms. In two other investigations the X-ray structure of rat m-calpain with calpastatin inhibitory domain 1 [16] and with calpastatin inhibitory domain 4 [17] has been determined. In both cases the position and length of the α-helices corresponding to calpastatin subdomains A and C in the bound form are in good agreement with the helix-forming tendencies of the corresponding calpA and calpC peptide segments we found in the free state (Figure 5). We note that similar to the conserved regions in subdomains A and C, a tendency for ordering has been observed for a segment of subdomain B which has been found to assume a type I β-turn in the free state [29].

Partial folding in the nascent state has already been suggested for a number of IUPs. For example, NMR studies have revealed that the *C*-terminal half of the mostly unfolded FlgM, a negative regulator of flagella and chemotaxis genes in bacteria, exists in an equilibrium structural state favoring helical conformations, which upon binding to its target becomes rigidly held [31]. Similar observations have been made for the cell-cycle inhibitor $p27^{Kip1}$ [32], and the CREB and p53 transactivator domains [33,34]. All these examples seem to follow a generalizable pattern that IUPs are not completely structureless in their free state but rather are structurally primed for the interaction with their target.

The Effect of Ca^{2+} on the Conformation of calpA and calpC

Biochemical studies have shown that calpastatin recognizes only the Ca^{2+}-bound state of calpain and the ability of calpastatin to distinguish between the inactive and the Ca^{2+}-activated conformations of calpain is maintained within subdomains A and C [21]. It has been the subject of interest whether calpastatin itself binds Ca^{2+} and whether this interaction is specific. Previously we have shown by CD spectroscopy that upon addition of Ca^{2+}, the helicity adopted by calpA and calpC in the presence of TFE becomes lost [35]. To monitor the effect of Ca^{2+} on a single residue level in the two peptides, we incubated them in CD_3OH/H_2O (1 : 1, v/v) with increasing amounts of $CaCl_2$. The amide proton regions of calpA and calpC in the absence and in the presence of two molar equivalents of Ca^{2+} are shown in Figures 6A and 6B. In both peptides, the most pronounced changes occur near the *C*-terminus at or near the Thr residue. In addition, resonance shifts of 0.05–0.1 ppm occur at Leu_{10} in calpA and throughout the chain of calpC (Ile_4, Gly_5, Asp_7, Ala_9, Ile_{10}, Asp_{11}). This suggests that in addition to the likely direct effects of Ca^{2+} at the acidic residues capable of Ca^{2+} coordination (e.g. Thr) there are also backbone rearrangements in the peptides. This is also supported by the observed H_α shift changes at hydrophobic residues in calpA (Ala_9, Ile_{14}, Suppl. Inf.). In fact, ROESY spectra of calpA and calpC acquired in 50% methanol with two molar equivalents of Ca^{2+} show a significantly less number of crosspeaks and with the exception of a short segment of a 3_{10} helix in the *N*-terminal half of calpA, structure calculations indicate a lack of well-defined structure for both peptides. The Ca^{2+}-induced destabilization of the secondary structure of calpA and calpC is in accordance with the earlier CD spectroscopy data [35] and is consistent with general models of Ca^{2+} binding in which side-chain and backbone (carbonyl) donating groups join together assuming a loop or turn conformation wrapped around the ion [36].

Intriguingly, although the fully activated calpain binds ten Ca^{2+} ions, calpastatin itself does not coordinate Ca^{2+} in the calpain–calpastatin complex [16]. Rather, it has been hypothesized that upon calcium-binding calpain undergoes a conformational change leading to activation and the binding of calpastatin [16,17,37]. Our investigation of calpA and calpC indicates that in their nascent states both peptides bind calcium and that Ca^{2+}-binding leads to the destabilization of the helices.

Conclusions

IUPs are functional polypeptide chains with an almost complete lack of a folded structure and high flexibility. Despite their random coil-like conformation, they are involved in the regulation of key cellular processes such as gene expression, signal transduction, and cell-cycle progression. By taking a direct advantage of their disordered nature, some of them function as entropic chains, whereas the majority of them exert their action via molecular recognition [19]. These interactions usually involve binding to other proteins or nucleic acid polymers and are accompanied by a disorder–order transition. To make the usually highly specific binding interactions of IUPs reversible and efficient, a balance between order and disorder is required before binding occurs [18,19].

Heat capacity and CD spectroscopy measurements on pig and human calpastatin domain 1 have suggested the presence of

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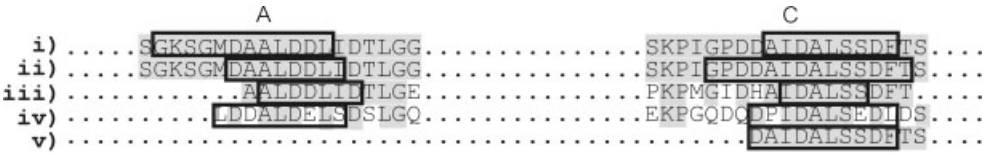

Figure 5. Sequential and topological alignments of calpA and calpC peptides with corresponding segments of calpastatin. (i) calpA and calpC[this study]; (ii) human calpastatin inhibitory domain 1 [30]; iii) rat calpastatin inhibitory domain 1 crystallized with calpain 2 [16]; iv) rat calpastatin inhibitory domain 4 crystallized with calpain 2 [17]; v) 12-residue segment from pig calpastatin inhibitory domain 1 crystallized with domain VI of calpain [28]. Identical residues are highlighted in *gray*. Alpha-helical segments are boxed.

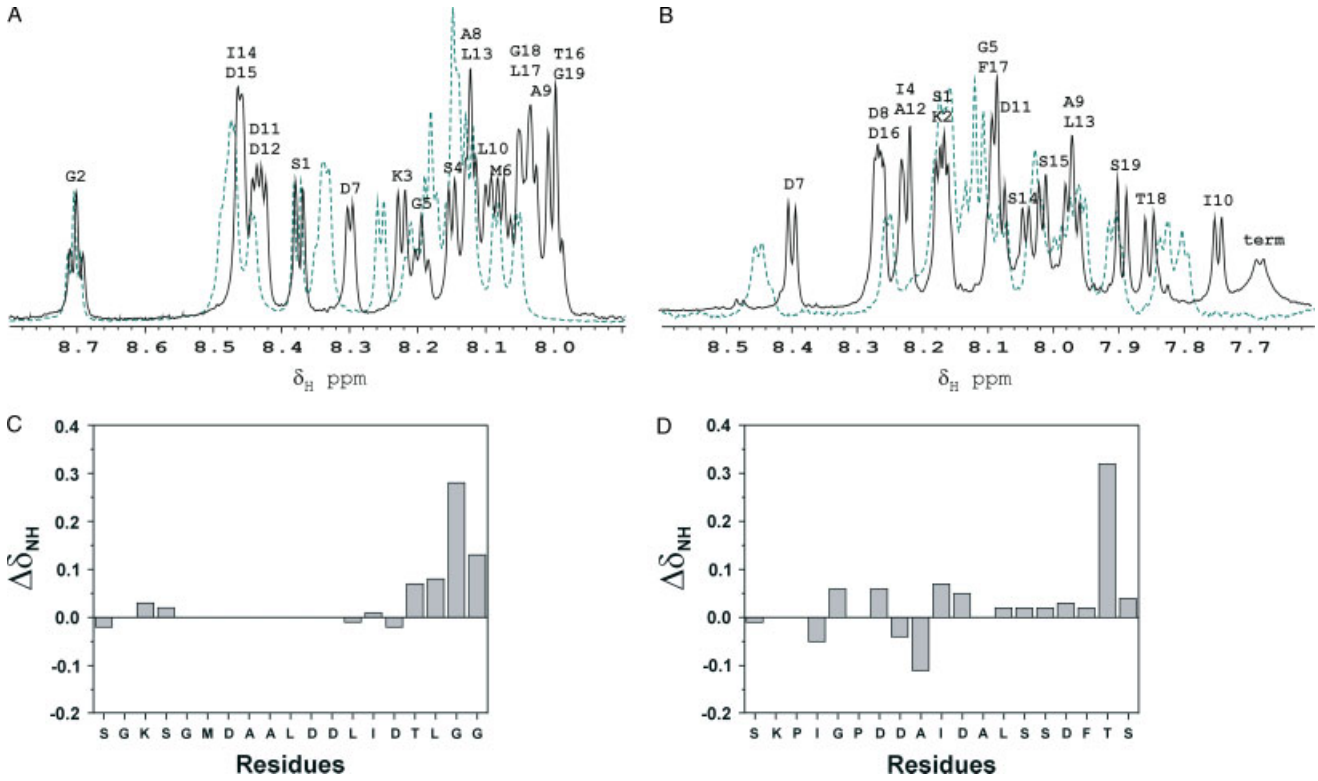

Figure 6. Calcium-induced changes in the NMR spectra of calpA and calpC. The amide region of 1D ^{1}H spectra of (A) calpA and (B) calpC in CD_3OH/H_2O (1 : 1, v/v) in the absence (*black solid*) and in the presence (*green dotted*) of two molar equivalents of $CaCl_2$. For simplicity, assignments are shown only for the spectra acquired in the absence of Ca^{2+}. Calcium-induced chemical shift changes of the amide protons in (C) calpA and (D) calpC.

residual structure in the inhibitor in its free state [38]. Our finding that calpA and calpC adopt an α-helical structure in 50% methanol suggests that there are segments in the conserved subdomains of calpastatin with an intrinsic tendency to assume the conformation of the bound form. The position and length of the helices in the two peptides matches the secondary structural elements in the bound calpastatin and are in agreement with a recently proposed binding model in which the disordered inhibitor wraps around calpain contacting it at distinct points [16,17,30]. The role of Ca^{2+} in calpain activation and calpain–calpastatin recognition is not fully understood yet. The destabilization of helices and the lose coordination of Ca^{2+} by the peptides fits into the model in which upon binding to calpain the conserved segments of calpastatin undergo a structural ordering and the coordination of Ca^{2+} becomes taken over by residues of the enzyme. Coordination of Ca^{2+} to calpastatin in its free, disordered state and to calpain in the enzyme-inhibitor complex may put Ca^{2+} into the position of a fine regulator that by opposing the conformational transition accompanying the interaction with calpain controls the binding event.

Acknowledgements

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Supporting information

Supporting information may be found in the online version of this article.

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