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Structure of the active 27-residue fragment of human calpastatin

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A synthetic 27-residue peptide corresponding to exon 1B of the endogenous inhibitor calpastatin contains a well-conserved region and has an ability to inhibit the cysteine endopeptidase calpain specifically. We examined the solution structure of this peptide in DMSO-d_6 by two-dimensional ^{1}H NMR spectroscopy. Although regular secondary structures such as α-helix and β-sheet were not found, the region from Ile^{18} to Arg^{23} formed a well-defined structure with a type I β-turn. This region coincided well with the highly conserved region of calpastatin. The result strongly suggests that this turn structure is essential for the inhibitory activity of calpastatin.

Human calpastatin; Calpain; ^{1}H NMR; Structure in solution

1. INTRODUCTION

The cysteine protease calpain (EC 3.4.22.17) is strongly and specifically inhibited by the endogenous protein calpastatin. Though both calpain and calpastatin are known to be common in animal cells [1–3] and are expected to play important roles in various cellular functions coupled with calcium-ion mobilization [4–8], nothing is known about how they interact with each other.

Calpastatin with about 700 residues contains four internally repetitive sequences (Domains 1–4) and one non-homologous sequence at the amino-terminal end (Domain L), each consisting of approximately 140 residues [9–11]. Each domain has three well-conserved regions located on separate exons, A,B and C, of which exon B is essential and strongly inhibits calpains I and II [12]. So far, no X-ray crystallography has been carried out on calpastatin or its domains. CD and ^{1}H NMR studies of Uemori et al. [13] strongly suggested that the repetitive domain is disordered in aqueous solution. Available knowledge of serine protease inhibitors [14] and also of cystatin, a cysteine protease inhibitor the crystal structure of which has recently been determined [15], indicate that certain well-defined structures are required for these protease inhibitors to bind specifically to target enzymes. The reported lack of well-defined structure for the repetitive domain of calpastatin [13] is rather puzzling from this viewpoint.

In the present work, using ^{1}H NMR, we studied the conformation of a synthetic oligopeptide of 27 residues corresponding to exon B in Domain 1 (exon 1B) in DMSO-d_6. This peptide contains the highly conserved region and retains a strong inhibitory potency against calpain ($K_i \simeq 10^{-8}$ M) in spite of its size constituting only one-fifth of the repetitive domain [16]. It is natural to expect that this indispensable peptide fragment will assume a certain defined structure at least when it binds to calpain. We chose DMSO-d_6 as a solvent, expecting that, to some extent, it will represent a somewhat hydrophobic environment for the inhibitor binding site.

Abbreviations: CD, circular dichroism; ^{1}H NMR, proton nuclear magnetic resonance; DMSO-d_6, dimethyl sulfoxide-d_6; HOHAHA, homonuclear Hartmann Hahn spectroscopy; DQFCOSY, double quantum filtered correlated spectroscopy; NOESY, nuclear Overhauser effect spectroscopy.

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2. MATERIALS AND METHODS

The synthesis and purification of the 27-residue peptide was performed as described previously [16]. For ^{1}H NMR measurements, the purified peptide was dissolved in DMSO-d_6 at a concentration of 5 mg/ml. One-dimensional spectral features in DMSO-d_6 showed little difference from those in ^2H_2O.

Two-dimensional DQFCOSY, HOHAHA (τ_{mixing}=100 and 120 ms) and NOESY (τ_{mixing}=50, 150, 300 and 500 ms) measurements were performed at 37°C on a JEOL GX-400 spectrometer with a spectral width of 5000 Hz and 2048 data points. Sixty-four scans were accumulated for each 256 column data under pre saturation of residual H^2HO.

3. RESULTS

Sequence-specific ^{1}H NMR assignments [17] were carried out by a combined use of DQFCOSY, HOHAHA and NOESY, and the results are summarized in

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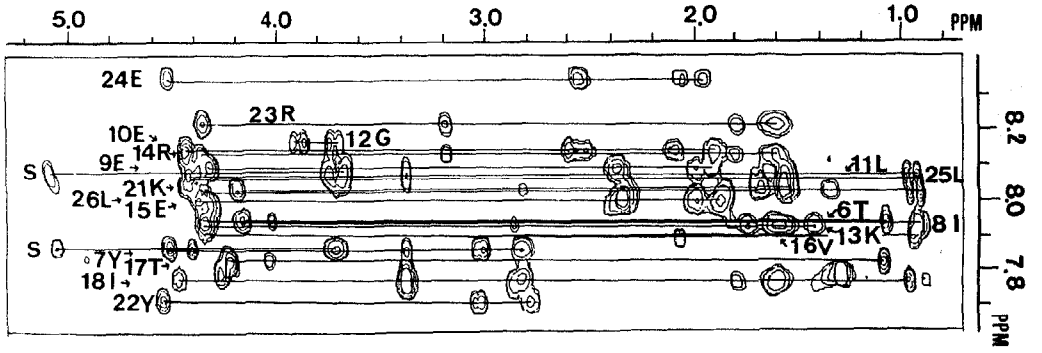

Fig. 1. A 2D HOHAHA spectrum (τ_{mix}= 120 ms) between the NH (7.6–8.4 ppm) and aliphatic protons (0.3–5.3 ppm) of the 27-residue peptide at 400 MHz. Specific signal assignments were obtained from sequential assignment.

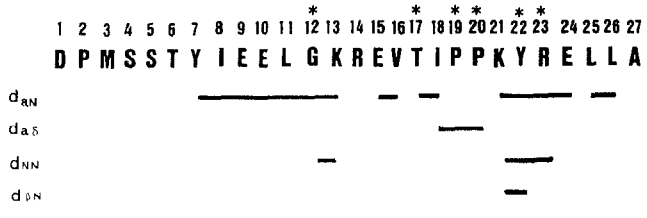

Fig. 2. Summary of the assigned inter-residue NOE connectivities (—). The highly conserved residues are marked with asterisks(*).

Table I. For residues Asp[1]–Ser[5], signal overlap and small NOE's did not allow reliable sequential assignments. Fig. 1 shows a HOHAHA spectrum for the aliphatic NH region. From studies with varied mixing time from 50 ms to 500 ms, 23 inter-residue NOE's (including sequential ones) could be identified as summarized in Fig. 2.

A notable feature in Fig. 2 is the presence of $H_{\alpha i}$–$H_{\delta i+1}$ NOE connectivities between Ile[18] and Pro[19] and between Pro[19] and Pro[20], which are derived from the spectrum shown in Fig. 3A. No $H_{\alpha i}$–$H_{\alpha i+1}$ NOE connectivities were identified for these residues. These results indicate that the conformations of the two consecutive peptide bonds, Ile[18]–Pro[19] and Pro[19]–Pro[20], are locked to *trans* [17]. Another notable feature of Fig. 2 is the presence of strong H_{Ni}–H_{Ni+1} NOE connectivities between Lys[21] and Tyr[22] and between Tyr[22] and Arg[23] (cf. Fig. 3B). In addition, N_i–β_{i+1} NOE was observed between Lys[21] and Tyr[22]. The observed inter-residue NOE's, together with a relatively large coupling constant $J_{N\alpha}$ for Tyr[22] (7.8 Hz), suggest strongly that the peptide segment of Pro[20]–Lys[21]–Tyr[22]–Arg[23] assumes a type I β-turn [17] with a hydrogen bonding between C=O of Pro[20] and NH of Arg[23].

Another strong H_{Ni}–H_{Ni+1} NOE connectivity was found between peaks at 8.14 ppm (assignable to Gly[12]) and 7.91 ppm (assignable to Lys[13]) (cf. Fig. 3B). This

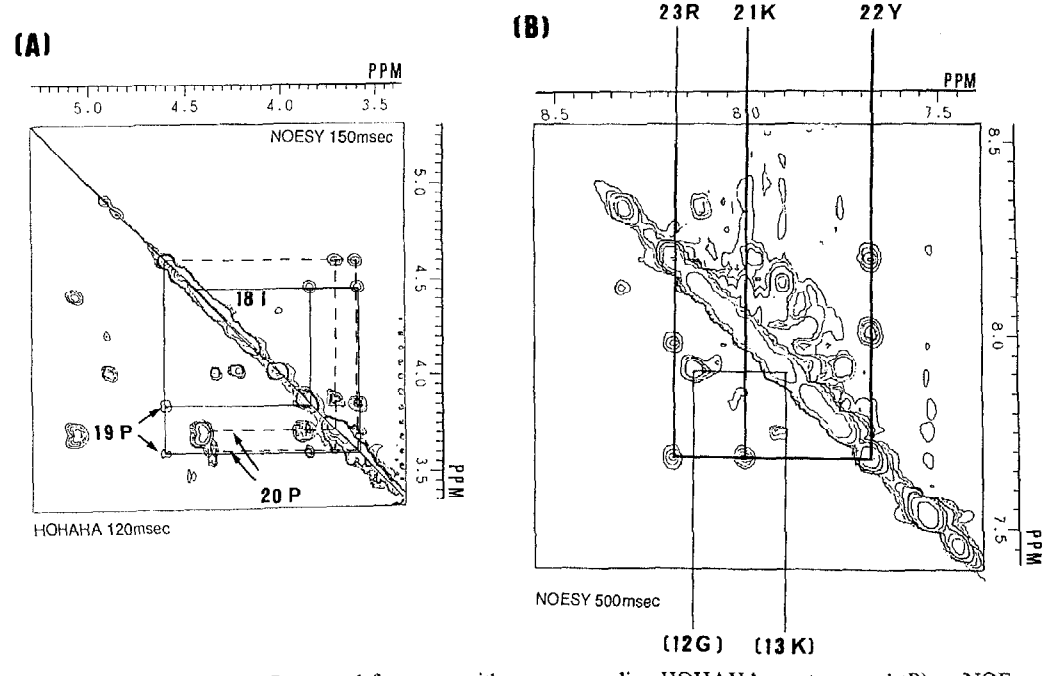

Fig. 3. (A) An NOE spectrum between the Pro α and δ protons with a corresponding HOHAHA spectrum and (B) an NOE spectrum between the NH protons, all measured at 400 MHz.

Table I

Summary of assigned ^{1}H chemical shifts of the 27-residue peptide having the sequence of exon 1B of calpastatin

Residue	Chemical shifts (ppm)					
	NH	α	β	others		
6 T	7.75	4.22	4.02	OH	4.90	
			4.02			
7 Y	7.83	4.51	3.02	δ	7.08	7.08
			2.81	ε	7.38	7.38
8 I	7.93	4.15	1.83	γCH3	0.89	0.89
				γCH3	1.51	1.18
				δCH3	0.90	
9 E	8.03	4.31	1.99	γ	2.35	2.36
			1.90	COOH	12.8	
10 E	8.12	4.44	2.11	γ	2.50	2.59
			1.90	COOH	12.8	
11 L	8.04	4.30	1.55	γ	1.55	
			1.55	δ	0.95	0.90
12G	8.14	3.74				
		3.86				
13 K	7.91	4.36	1.78	γ	1.40	1.41
			1.78	δ	1.61	1.61
				ε	7.72	
14 R	8.12	4.34	1.80	γ	1.59	1.61
			1.80	δ	3.19	3.19
				ε NH	7.51	
15E	7.96	4.37	1.88	γ	2.35	2.35
			1.97			
				COOH	12.8	
16 V	7.98	4.35	1.70	γ	0.95	0.93
17 T	7.89	4.34	4.01	OH	4.90	
			4.01			
18 I	7.73	4.47	1.79	γCH3	0.90	0.87
				γCH2	1.58	1.13
				δCH3	0.95	
19 P		4.62	2.22	γ	1.89	1.90
			1.99	δ	3.85	3.61
20 P		4.40	2.10	γ	1.97	1.88
			1.98	δ	3.72	3.61
21 K	8.01	4.19	1.68	γ	1.33	1.34
			1.68	δ	1.56	1.56
				ε	2.81	2.81
				εNH2	7.72	
22 Y	7.67	4.53	2.79	δ	7.08	7.08
			3.02	ε	7.38	7.38
23 R	8.19	4.34	1.80	γ	1.59	1.61
			1.80	δ	3.19	3.19
				εNH	7.51	
24 E	8.32	4.52	2.18	γ	2.56	2.56
			1.97	COOH	12.8	
25 L	8.00	4.4	1.55	γ	1.55	
			1.55	δ	0.89	0.94
26 L	7.86	4.34	2.25	γ	2.05	
			2.05	δ	0.93	0.93
27 A	7.75	4.26	1.38			

indicates a possibility of another turn (Type II) [17] involving Glu[10]-Leu[11]-Gly[12]-Lys[13].

4. DISCUSSION

Regular secondary structures such as α-helix and β-sheet were not found for the active 27-residue peptide. The result appears to be consistent with the previous study on Domain 1 [13]. However, the region from Ile[18] to Arg[23] was clearly shown to have a restricted structure in which the conformations of the two consecutive peptide bonds, Ile[18]–Pro[19] and Pro[19]–Pro[20], are locked to *trans*, and Pro[20]–Lys[21]–Tyr[22]–Arg[23] forms a type I β-turn. A most interesting point is that this region coincides almost perfectly with the region of the peptide Thr[17]–Arg[23] consisting of highly conserved residues (marked with asterisks in Fig. 2). A possibility of another turn was suggested for the region from Glu[10] to Lys[13], which also contains a highly conserved residue, Gly[12]. These findings suggest strongly that the restricted structures formed by Ile[18]–Arg[23], and probably also by Glu[10]–Lys[13], are essential for the specific interaction of calpastatin with calpain. From the present study combined with the earlier results by Uemori et al. [13], we may conclude that, although the repetitive domain of calpastatin does not have an extensive tertiary structure such as those found in most serine protease inhibitors [14] and cystatin [15], it would have a well-defined local structure essential for the specific interaction with calpain.

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