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Natural abundance ^{15}N NMR assignments delineate structural differences between intact and reactive-site hydrolyzed *Cucurbita maxima* trypsin inhibitor III

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^{15}N NMR assignments were made to the backbone amide nitrogen atoms at natural isotopic abundance of intact and reactive-site (Arg^5–Ile^6) hydrolyzed *Cucurbita maxima* trypsin inhibitor III (CMTI-III and CMTI-III*, respectively) by means of 2D proton-detected heteronuclear single bond chemical shift correlation (HSBC) spectroscopy, utilizing the previously made sequence-specific ^1H NMR assignments (Krishnamoorthi et al. (1992) Biochemistry 31, 898–904). Comparison of the ^{15}N chemical shifts of the two forms of the inhibitor molecule revealed significant changes not only for residues located near the reactive-site region, but also for those distantly located. Residues Cys^3, Arg^5, Leu^7, Met^8, Cys^{10}, Cys^{16}, Glu^{19}, His^{25}, Tyr^{27}, Cys^{28} and Gly^{29} showed significant chemical shift changes ranging from 0.3 to 6.1 ppm, thus indicating structural perturbations that were transmitted throughout the molecule. These findings confirm the earlier conclusions based on ^1H NMR investigations.

Inhibitor; Serine protease; *Cucurbita maxima*; Pumpkin; Trypsin; Activated Hageman factor; Blood coagulation; ^1H–^{15}N chemical shift correlation

1. INTRODUCTION

Cucurbita maxima trypsin inhibitor (CMTI's) are small globular proteins (M_r 3 kDa) belonging to the recently characterized squash inhibitor family [1,2]. They each contain 29 to 32 amino acid residues, including three disulfide bridges. CMTI-I and CMTI-III differ from each other by the single amino acid substitution of Glu^9 in the former by a Lys in the latter. While an X-ray crystallographic structure is available for CMTI-I complexed with trypsin [3], a solution NMR structure exists for the free inhibitor molecule [4]. The structural data reveal that the secondary structure of CMTI-I consists mainly of two tight turns, a 3_{10}-helix, and a triple-stranded sheet. The reactive-site has been established to be the peptide bond between Arg^5 and Ile^6 [1]. Reaction of an intact CMTI with a serine proteinase such as trypsin results in the formation of a reactive-site hydrolyzed (modified) inhibitor, CMTI*, in which the two fragments are held together by a disulfide bond.

Abbreviations: CMTI, *Cucurbita maxima* trypsin inhibitor; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; HSBC, heteronuclear single bond chemical shift correlation spectroscopy; ppm, parts per million.

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While the intact inhibitors inhibit both trypsin and activated Hageman factor, a blood coagulation protein, the modified forms inhibit only trypsin but not activated Hageman factor [5,6]. In an effort to characterize the structural changes induced by hydrolysis of the reactive-site peptide bond, we recently carried out a 2D NMR investigation of modified CMTI-III and established that while the secondary structural elements were preserved in CMTI-III*, tertiary structural changes occurred throughout the molecule [7].

^{15}N NMR spectroscopy offers a particularly attractive tool for monitoring the changes in structures and dynamics of proteins [8,9]. To date two-dimensional ^1H–^{15}N chemical shift correlation spectroscopy has been mainly applied to a number of large proteins that have been labeled with ^{15}N at the backbone amide nitrogens [10]. A few examples exist in which the NMR experiments were performed at natural isotopic abundance [11–13]. CMTI-III, being small in size, has permitted the application of ^{15}N NMR at natural isotopic abundance. Here we report the ^{15}N NMR assignments of the backbone amide nitrogen atoms of CMTI-III and CMTI-III*, as obtained by proton-detected 2D heteronuclear single bond chemical shift correlation spectroscopy (HSBC). Comparison of the assigned chemical shifts of the two forms of the inhibitor molecule reveals that not only residues located near the reactive region, but also those located far away are perturbed by the cleavage of the scissile bond. These results confirm our earlier work involving ^1H NMR chemical shifts.

2. MATERIALS AND METHODS

2.1. *Proteins*

CMTI-III and CMTI-III* were isolated from pumpkin seeds by means of trypsin affinity chromatography and reverse-phase high-performance liquid chromatography (HPLC), as described earlier [5]. The separated protein fractions were repeatedly submitted to HPLC to achieve homogeneity. CMTI-III was also produced by reacting CMTI-III* with trypsin and separating the products by means of HPLC. An NMR sample was typically prepared by dissolving about 30 mg of the protein in 0.5 ml of water containing 10% ^2H_2O (by volume). The sample also contained 0.2 M KCl. The pH of the NMR sample was adjusted to 4.71 at 30°C with 0.2 M KOH or 0.2 M HCl, using an Ingold microcombination glass electrode and a Fisher (model 815 MP) pH meter.

2.2. *NMR spectroscopy*

A 500 MHz GE Omega NMR spectrometer was employed to collect 2D ^1H–^{15}N chemical shift correlation data. Proton-detected HSBC experiments were performed. Data were collected with and without ^{15}N decoupling, employing standard pulse sequences and phase-cycling schemes [14]. ^{15}N decoupling was achieved by means of the WALTZ16 pulse scheme [15]. The decoupler power was 5 W. The water peak was saturated continuously during the relaxation delay with the low power transmitter. Typically, a 2D data set was acquired by collecting 128 t_1 blocks of free induction decays containing 1024 data points each. Each block contained 128 acquisitions in the case of CMTI-III* and 400 in the case of CMTI-III. The 2D data sets which were acquired without ^{15}N decoupling contained 800 acquisitions per block in the case of CMTI-III, and 128 acquisitions per block in the case of CMTI-III*. A proton spectral width of 6,600.66 Hz in the t_2 dimension and a nitrogen spectral width of 2,500 Hz in the t_1 dimension were used. The t_1 dimension was zero-filled four times, and the t_2 dimension once. A 90° phase-shifted sinesquared window function was employed in both the dimensions. A ^1H–^{15}N chemical shift correlation 2D NMR experiment took approximately 30 h in the case of CMTI-III, and 65 h in the case of CMTI-III*. The reported ^{15}N chemical shifts are relative to a value of 118.71 ppm for acetamide in ^1H_2O at 30°C.

3. RESULTS

The ^1H–^{15}N chemical shift correlation spectra (HSBC) of CMTI-III and CMTI-III* at pH 4.71, 30°C are shown in Figs. 1 and 2, respectively. ^{15}N decoupling was employed to remove the single bond scalar coupling between ^{15}N and ^1H. As a result, only a single cross peak appears for each of the peptide N–H pairs. Splitting of each proton signal due to spin coupling with $C_\alpha H$ is visible in the proton dimension. The proton NMR spectra of both CMTI-III and CMTI-III* under similar conditions of pH and temperature have been previously assigned [7]. It was, therefore, straightforward to assign the cross peaks in Fig. 1 by simple chemical shift correlation. No difficulty was experienced in assigning any of the existing cross peaks, as they were well-resolved and no problem due to overlap was encountered. The ^{15}N chemical shifts determined for the backbone amide nitrogens of CMTI-III and CMTI-III* are collected in Table I. The free amino groups of CMTI-III (Arg^1) and CMTI-III* (Arg^1 and Ile^6) could not be observed due to a rapid exchange with solvent water molecules, which were presaturated with a low power pulse. Weak cross peaks assigned to Val^2 and Met^8 in CMTI-III (Fig. 1)

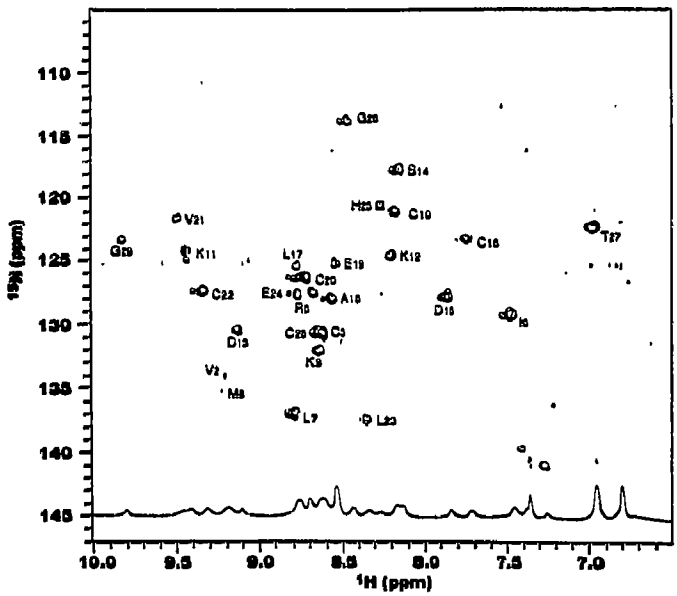

Fig. 1. ^1H–^{15}N chemical shift correlation map of CMTI-III at pH 4.71, 30°C. ^{15}N assignments are obtained by cross correlation with proton assignments [7].

were confirmed by the presence of relatively stronger peaks in the ^1H–^{15}N chemical shift correlation spectrum obtained without ^{15}N decoupling (not shown). Similarly, the His^{25} cross-peak position (Fig. 1) was determined from the pair of cross peaks found in the correlation spectrum collected without ^{15}N decoupling. Poor S/N in both coupled and decoupled spectra precluded identification of a cross peak due to amide ^{15}N–^1H of Val2 of CMTI-III*.

4. DISCUSSION

^{15}N NMR chemical shifts serve as powerful probes to detect structural changes. They are especially sensitive to changes in hydrogen bonding interactions [16]. ^{15}N chemical shifts have also been demonstrated to be sensitive to nonbonded interactions [13]. Solvent effects on hydrogen bonding interactions have been well-characterized by ^{15}N NMR spectroscopy [16,17]. In the case of CMTI-III and CMTI-III*, a comparison of the ^{15}N chemical shifts (Fig. 1 and Table I) indicate changes for many residues. Fig. 3 shows a plot of ^{15}N chemical shift differences between CMTI-III and CMTI-III* as a function of residue position. It is thus evident that residues Cys^3, Arg^5, Leu^7, Met^8, Cys^{10}, Cys^{16}, Glu^{19}, His^{25}, Tyr^{27}, Cys^{28} and Gly^{29} undergo chemical shift changes ranging from 0.27 to 6.08 ppm. The greatest shift change is observed for Arg^5, which becomes free and exposed to more solvent interaction in CMTI-III*. From proton NMR investigation we earlier established that residues Val^2, Cys^3, Arg^5, Ile^6, Leu^7, Met^8, Lys^9, Lys^{11}, Asp^{13}, Asp^{15}, Cys^{16}, Leu^{17}, Ala^{18}, Cys^{20}, Val^{21}, Cys^{22}, Leu^{23}, His^{25}, Gly^{26}, Tyr^{27}, Cys^{28}, and Gly^{29} were

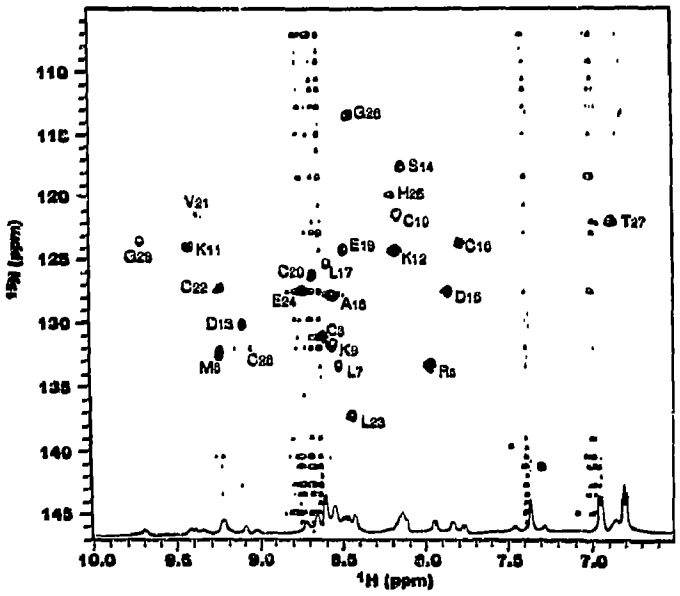

Fig. 2. ^{1}H–^{15}N chemical shift correlation map of CMTI-III* at pH 4.71, 30°C. ^{15}N assignments are obtained by cross correlation with proton assignments [7].

Table 1

^{15}N chemical shifts of backbone amide nitrogens of CMTI-III and CMTI-III* at pH 4.71, 30°C

Residue	CMTI-III	CMTI-III*
Arg^{1}	–	–
Val^{2}	134.1	–
Cys^{3}	130.7	131.3
Pro^{4}	–	–
Arg^{5}	127.4	133.5
Ile^{6}	129.2	–
Leu^{7}	136.9	133.5
Met^{8}	135.3	132.6
Lys^{9}	132.0	131.9
Cys^{10}	120.9	122.1
Lys^{11}	124.1	124.1
Lys^{12}	124.5	124.4
Asp^{13}	130.4	130.3
Ser^{14}	117.6	117.8
Asp^{15}	127.8	127.7
Cys^{16}	123.1	123.8
Leu^{17}	125.3	125.5
Ala^{18}	127.9	127.9
Glu^{19}	125.2	124.3
Cys^{20}	127.5	127.6
Val^{21}	121.5	121.7
Cys^{22}	127.3	127.4
Leu^{23}	137.4	137.5
Glu^{24}	126.2	126.3
His^{25}	120.3	119.9
Gly^{26}	113.7	113.6
Tyr^{27}	121.8	122.1
Cys^{28}	130.6	132.1
Gly^{29}	123.3	123.6

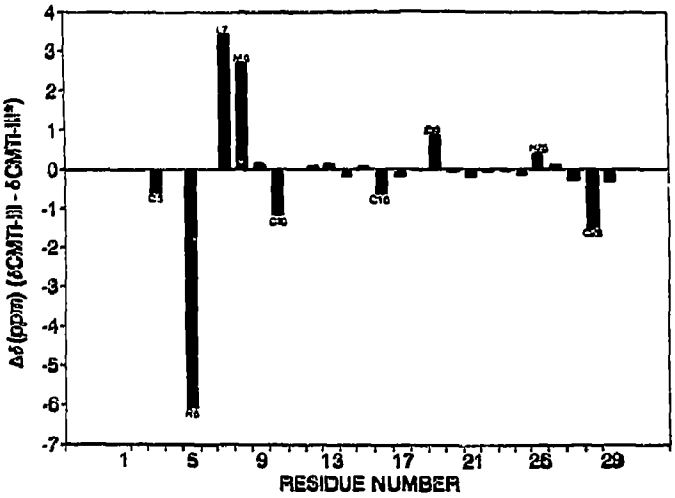

Fig. 3. Plot of differences in ^{15}N chemical shifts of CMTI-III and CMTI-III* as a function of residue number.

all affected by hydrolysis of the reactive-site peptide bond [7]. However, ^{15}N chemical shifts of CMTI-III and CMTI-III* (Table I; Fig. 2) reveal that the first eight N-terminal residues and those that form secondary structural elements show significant perturbations. The X-ray structure of CMTI-I reveals that residues Leu^{17} to Cys^{20} and Leu^{23} to Gly^{26} form two tight turns [3]. The N-terminal residues that are affected by cleavage of the Arg^{5}–Ile^{6} peptide bond form part of a hydrophobic surface in the intact molecule. Furthermore, the X-ray structure indicates involvement of the following residues in hydrogen bonding interactions [3]: Met^{8}, Cys^{10}, Lys^{12}, Asp^{13}, Asp^{15}, Cys^{16}, Val^{21}, Leu^{23}, Gly^{26}, Tyr^{27}, Cys^{28} and Gly^{29}. Thus, the ^{15}N chemical shift changes (Table I; Fig. 3) observed for CMTI-III* could arise either from changes in solvent interactions or from inter-residue hydrogen bonding interactions. Deuterium-exchange kinetics of peptide NH's did not reveal significant differences between CMTI-III and CMTI-III* on the time scale involved in the measurements (h) [7]. The assignments reported here could be used to study the relaxation behavior and heteronuclear Overhauser effect in CMTI-III and CMTI-III* to derive information on backbone dynamics [18].

The thermodynamic measurements of the equilibrium between CMTI-III and CMTI-III* at 30°C indicate that the modified form is more stable, $\Delta G°$ being about −600 cal/mol and $\Delta S°$ −8.89 e.u. (Krishnamoorthi et al., Biochemistry, in press). Interpretation of thermodynamic parameters critically depends upon structural knowledge. The present investigation confirms our previous conclusion based on ^{1}H NMR work that structural changes are not confined to the reactive-site region of the inhibitor molecule, but are transmitted throughout the molecule [7]. While it is not possible to interpret changes in chemical shifts in terms of specific

structural changes, it is clear that structural changes occurring as a result of hydrolysis of the reactive-site peptide bond result in greater stability of the inhibitor molecule, perhaps through more extensive solvent interactions. The loss of entropy accompanying hydrolysis of the scissile peptide bond suggests a more ordering of solvent water molecules around the modified inhibitor. It is of interest to note that the residues that are identified as being perturbed due to cleavage of the reactive-site peptide bond are known to be involved in protein-protein interaction in the trypsin:inhibitor complex, as determined by X-ray crystallography [3]. The present observation with CMTI-III* is in contrast to the results obtained with other larger serine proteinase inhibitors, namely, modified ovomucoid third domain inhibitors, in which cases the hydrolysis of the reactive-site bond causes structural changes confirmed mainly to the reactive-site region [19,20].

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