Structural Consequences of the Natural Substitution, E9K, on Reactive-Site-Hydrolyzed Squash (*Cucurbita maxima*) Trypsin Inhibitor (CMTI), As Studied by Two-Dimensional NMR†

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ABSTRACT: Sequence-specific hydrogen-1 NMR assignments were made to all of the 29 amino acid residues of reactive-site-hydrolyzed *Cucurbita maxima* trypsin inhibitor I (CMTI-I*) by the application of two-dimensional NMR (2D NMR) techniques, and its secondary structural elements (two tight turns, a 3_{10}-helix, and a triple-stranded β-sheet) were identified on the basis of short-range NOESY cross peaks and deuterium-exchange kinetics. These secondary structural elements are present in the intact inhibitor [Holak, T. A., Gondol, D., Otlewski, J., & Wilusz, T. (1989) *J. Mol. Biol. 210*, 635–648] and are unaffected by the hydrolysis of the reactive-site peptide bond between Arg5 and Ile6, in accordance with the earlier conclusion reached for CMTI-III* [Krishnamoorthi, R., Gong, Y.-X., Lin, C. S., & VanderVelde, D. (1992) *Biochemistry 31*, 898–904]. Chemical shifts of backbone hydrogen atoms, peptide N*H*'s, and $C_\alpha H$'s, of CMTI-I* were compared with those of the intact inhibitor, CMTI-I, and of the reactive-site-hydrolyzed, natural, E9K variant, CMTI-III*. Cleavage of the Arg5–Ile6 peptide bond resulted in changes of chemical shifts of most of the backbone atoms of CMTI-I, in agreement with the earlier results obtained for CMTI-III. Comparison of chemical shifts of backbone hydrogen atoms of CMTI-I* and CMTI-III* revealed no changes, except for residues Glu9 and His25. However, the intact forms of the same two proteins, CMTI-I and CMTI-III, showed small but significant perturbations of chemical shifts of residues that made up the secondary structural elements of the inhibitors. Apparently, CMTI-I loses its conformational sensitivity to the E9K substitution upon conversion to CMTI-I*. In order to gain an insight into the relative stabilities of the intact and reactive-site-hydrolyzed forms, the equilibrium between the intact and modified inhibitors was investigated by high-performance liquid chromatography (HPLC), and the following thermodynamic data were obtained for CMTI-III: $\Delta H° = -3.16 \pm 0.69$ kcal/mol; $\Delta S° = -8.89 \pm 0.37$ eu. $\Delta G°$ was determined to be -607 ± 18 cal/mol for the equilibrium between CMTI-III and CMTI-III* and -669 ± 19 cal/mol for the equilibrium between CMTI-I and CMTI-I* at 30 °C.

Pumpkin (*Cucurbita maxima*) seeds contain three small (3 kDa) protein inhibitors and one large (7 kDa) protein inhibitor of trypsin (Wieczorek et al., 1985; Krishnamoorthi et al., 1990; Otlewski, 1990). These proteins, termed CMTI-I,[1] CMTI-III, CMTI-IV, and CMTI-V, are also specific inhibitors of an activated blood coagulation protein, namely, Hageman factor or factor XII_a. CMTI-V does not bear any sequence similarities to the smaller proteins and belongs to the potato I inhibitor family (Krishnamoorthi et al., 1990). All of these protein inhibitors lose their ability to inhibit factor XII_a, but not trypsin, upon conversion into their modified forms in which the reactive-site peptide bond (Arg5–Ile6 for CMTI-I and CMTI-III; and Lys44–Asp45 in CMTI-V) has been hydrolyzed (Hojima et al., 1982; Krishnamoorthi et al., 1990). Variants of CMTI-III, including the naturally occurring protein, have been chemically synthesized in the solid phase and studied in an effort to understand the structure–function relationships in these smallest known globular trypsin inhibitors (Kupryszewski et al., 1986; McWherter et al., 1989). These studies have further been fueled by the availability of high-resolution X-ray crystal (Bode et al., 1989) as well as solution NMR structures of the native (intact) protein, CMTI-I (Holak et al., 1989a,b). Recently, we characterized by two-dimensional NMR the structural changes, as reflected by changes in chemical shifts, undergone by various amino acid residues in CMTI-III upon cleavage of the scissile bond between Arg5 and Ile6 (Krishnamoorthi et al., 1992a). We further compared the chemical shifts of backbone hydrogens in intact CMTI-I and CMTI-III, which differed from each other by the E9K substitution, and found that the substitution affected mainly those residues that constituted the secondary structural elements of the protein, whereas those in an extended conformation remained unaffected. According to the "standard mechanism" of inhibition of proteolytic enzymes (Laskowski & Kato, 1980), both native and reactive-site-hydrolyzed (modified) forms of an inhibitor form complexes with their cognate enzyme, and therefore, differential interactions between the intact and modified forms of the inhibitor with the

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[1] Abbreviations: CMTI, *Cucurbita maxima* trypsin inhibitor; 2D, two dimensional; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; TOCSY, total correlated spectroscopy; DQF-COSY, double-quantum-filtered correlated spectroscopy; NOESY, two-dimensional NOE spectroscopy; CMTI-I*, reactive-site-hydrolyzed CMTI-I; CMTI-III*, reactive-site-hydrolyzed CMTI-III.

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enzyme can lead to modulation of the enzyme's activity. Although general reports have appeared on the structures, dynamics, and functions of native protein inhibitors that differ from one another by a single amino acid substitution, to date, to our knowledge, no report has dealt with the effect of single amino acid substitution on the structure of the modified form of an inhibitor. Therefore, in order to characterize the effect of the single amino acid substitution, E9K, on the conformation of the reactive-site-hydrolyzed inhibitor, we have now extended our 2D NMR investigation to CMTI-I* and made sequence-specific hydrogen-1 assignments to its 29 amino acid residues. Comparison of chemical shifts of the backbone hydrogens of CMTI-I* and of CMTI-III* (Krishnamoorthi et al., 1992a) shows surprisingly negligible differences. Apparently, in its modified form, the inhibitor molecule loses sensitivity to this amino acid substitution. Thermodynamic parameters associated with the equilibrium between the intact and modified forms of CMTI-III were estimated from the temperature dependence of the equilibrium constant, K_{hyd}. The data reveal that the modified form of the inhibitor is thermodynamically more favorable.

Materials and Methods

Proteins. The protein inhibitors, CMTI-I* and CMTI-III*, were isolated from pumpkin seeds and purified, as described before (Krishnamoorthi et al., 1990). A sample for NMR studies was prepared by dissolving about 10 mg of the lyophilized protein in 0.4 mL of H_2O that contained 10% by volume ^2H_2O. The pH of the sample was adjusted with 0.2 M ^2HCl and/or 0.2 M KO^2H, using a Fisher pH meter equipped with an Ingold microcombination glass electrode.

NMR Spectroscopy. The following 2D NMR experiments were performed with a Bruker AM 500 instrument (500.14 MHz for ^1H), and the data were processed on a Silicon Graphics workstation, using the software FELIX (Hare Research Inc., Woodinville, WA): double-quantum-filtered COSY (DQF-COSY; Piantini et al., 1982; Rance et al., 1983); total correlated spectroscopy (TOCSY; Braunschweiler & Ernst, 1983) with an MLEV17 spin lock (Bax & Davis, 1985); and phase-sensitive nuclear Overhauser effect spectroscopy (NOESY; Anil Kumar et al., 1980). A mixing time of 70 ms was used for the TOCSY experiment. The NOESY experiment employed a mixing time of 200 ms. All data were collected by using the time-proportional phase incrementation (TPPI; Marion & Wuethrich, 1983). A typical data matrix consisted of 1024 × 1024 real points. Chemical shifts were referenced by assigning a value of 4.71 ppm at 30 °C to the water peak. For the purpose of locating $C_\alpha H$ peaks that occurred close to the water peak (those of Lys and Glu), TOCSY and NOESY data sets were acquired at 10 and 45 °C. Exchange of solvent-labile backbone N*H*'s with deuterated water was followed by collecting 1D NMR spectra at a series of time intervals of 10 min or less.

Determination of the Equilibrium Constant, K_{hyd}. K_{hyd}, defined as [I*]/[I] (Laskowski & Kato, 1980), where [I*] is the concentration of reactive-site-hydrolyzed inhibitor and [I] is the concentration of intact inhibitor at equilibrium, was determined by means of HPLC. About 5.5×10^{-7} mol of the inhibitor in 0.4 mL of H_2O containing 0.05 M sodium acetate at pH 4.71 was mixed with 2.4×10^{-8} mol of trypsin and equilibrated in a dry bath at 30 °C. After every 24-h period, 40 μL of the enzyme–inhibitor mixture was injected into a Varian HPLC that used a C-18 reverse-phase column and a gradient elution system: Solvent A consisted of 0.1% trifluoroacetic acid (TFA) in H_2O, and solvent B consisted of 0.1% TFA in acetonitrile. The composition varied from 80% solvent A to 75% solvent A in 25 min, when the inhibitors were eluted, followed by a change to 55% solvent A in 20 min, when trypsin came out of the column. Periodically, aliquots of the reaction mixture were withdrawn, and the relative proportions of the intact and modified forms of the inhibitor were determined by HPLC, until no change in the equilibrium constant so determined was observed. The equilibrium was attained within a period of 4 days at 30 °C. The same position of equilibrium was reached by starting with either intact or modified inhibitor. For each reaction, two HPLC injections were performed, five copies of each chromatogram were made, and peaks were cut out and weighed. The ratios of weights of peaks corresponding to intact and modified inhibitors were taken as a measure of their relative concentrations. For the temperature dependence study, equilibrating mixtures were kept in either a refrigerator or an oven whose temperature could be varied and maintained. Thus, K_{hyd} was determined at 2, 5, 30, 47, and 55 °C.

Table I: Chemical Shifts of the Assigned ^1H NMR Resonances of CMTI-I* at pH 4.71, 30 °C

residue	N*H*	$C_\alpha H$	$C_\beta H$	$C_\gamma H$	others
Arg1		4.14	1.90	1.59, 1.50	3.21, 3.28, 7.41
Val2	8.95	4.13	2.03	0.94, 0.90	
Cys3	8.55	5.10	2.84		
Pro4		4.47	2.25, 2.03	2.00	3.77, 3.72
Arg5	7.89	4.21	1.87, 1.74	1.67	3.24, 7.23
Ile6		3.89	1.97	1.49, 1.22, 1.01	0.98
Leu7	8.45	4.48	1.47	1.53	0.69, 0.62
Met8	9.15	4.60	2.08, 1.92	2.60	
Glu9	8.60	4.66	2.19, 2.00	2.42, 2.32	
Cys10	8.09	4.86	3.20, 3.09		
Lys11	9.34	4.43	1.87, 1.74	1.44	1.74, 3.01
Lys12	8.10	4.74	1.97, 1.82	1.39, 1.23	1.64, 2.97
Asp13	9.06	4.14	2.80, 2.72		
Ser14	8.08	4.34	4.13, 3.85		
Asp15	7.78	4.62	3.02, 2.98		
Cys16	7.69	4.85	2.98, 2.72		
Leu17	8.49	4.26	1.69, 1.53	1.53	0.94, 0.90
Ala18	8.51	4.00	1.49		
Glu19	8.41	4.43	2.27, 2.16	2.37	
Cys20	8.52	4.58	3.40, 3.16		
Val21	9.28	4.40	2.14	0.81, 0.72	
Cys22	9.15	4.84	2.81, 2.42		
Leu23	8.38	4.38	1.61	1.76	0.87, 0.78
Glu24	8.58	3.98	2.00, 1.98	2.14, 2.08	
His25	8.14	4.57	3.53, 3.36		8.58, 7.30
Gly26	8.37	4.00, 3.80			
Tyr27	6.80	5.45	2.97, 2.76		6.90, 6.75
Cys28	8.66	5.32	3.10, 2.91		
Gly29	9.64	4.14, 3.80			

Results and Discussion

*Sequence-Specific Assignments and Secondary Structure of CMTI-I**. Individual spin systems of CMTI-I* were identified in the COSY and TOCSY maps of CMTI-I*, and sequential assignments (Wuethrich, 1986) were made in combination with NOESY data, as described before for CMTI-III* (Krishnamoorthi et al., 1992a). Short stretches of assignments were made as follows (supplementary material): Gly29 → Val21; Cys20 → Cys16; Asp15 → Asp13; Lys11 → Ile6; Arg5 → Pro4; and Cys3 → Val2. Table I lists the chemical shift data of backbone and side-chain hydrogens of all of the 29 amino acid residues of CMTI-I* at pH 4.71, 30 °C. An analysis of all the observed NOESY cross peaks yields the diagonal plot (Figure 1) depicting backbone–backbone, backbone–side-chain, and side-chain–side-chain interactions. The d_{NN} cross peak observed at 9.34 and 8.10 ppm showed degeneracy at 30 °C, which was removed at 10 °C, resulting in its unambiguous assignment to Lys11 and Lys12. An analysis of NOESY cross peaks (Figure 1) leads to the identification of two tight turns involving residues 9–12 and

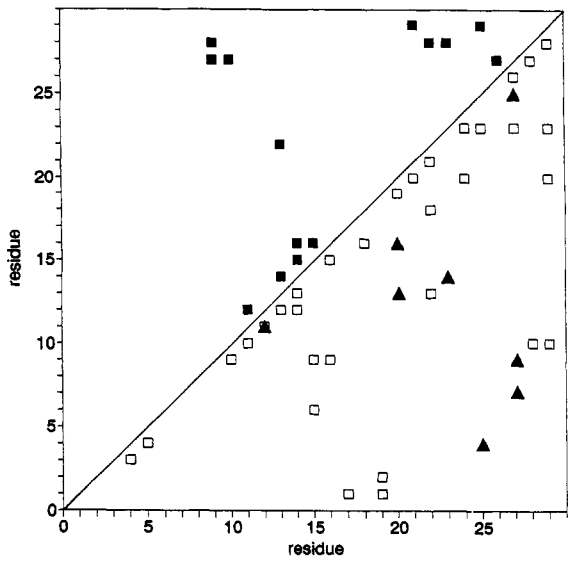

FIGURE 1: Diagonal plot of short-range NOE cross peaks obtained with a NOESY mixing time of 200 ms for CMTI-I* at pH 4.71, 30 °C. Observed backbone–backbone interactions (filled squares) are represented above the diagonal, whereas backbone–side-chain (open squares) and side-chain–side-chain (filled triangles) interactions are represented below the diagonal.

24–27, a 3_{10}-helix made up of residues 13–16, and a triple-stranded β-sheet involving residues 8–10, 29–27, and 21–25. The same secondary structural elements were earlier characterized for the intact inhibitor, CMTI-I, by Holak et al. (1989a). These secondary structural elements were further confirmed by the observation that the peptide amide hydrogens of residues Cys10, Ser14, Asp15, Cys16, Val21, Cys22, Leu23, Tyr27, Cys28, and Gly29 exhibited retarded exchange in the hydrogen–deuterium-exchange study. Thus, as found for CMTI-III*, the secondary structural elements of the intact inhibitor, CMTI-I (Holak et al., 1989a), are also preserved in CMTI-I*, in which the scissile bond between Arg5 and Ile6 has been hydrolyzed. However, differences between CMTI-I* and CMTI-III* occur in the pattern of cross peaks observed for side-chain–side-chain interactions. For example, the following cross peaks are observed only for CMTI-III* and not for CMTI-I*: C20β–C28β' and C20β–C28β. Similarly, the following cross peaks are observed only for CMTI-I* and not for CMTI-III*: L7(CH_3)–Y27δ; E9γ–Y27δ; K11ϵ–K12γ; D13β–C20β; S14β–L23(CH_3); H25β–P4δ; Y27δ,ϵ–K12γ. The conformational differences between CMTI-I* and CMTI-III* can be analyzed only when high-resolution three-dimensional structures are determined for these molecules by the application of a distance-geometry algorithm, in which NOE-derived distances are used as initial constraints (Havel & Wuethrich, 1985; Braun, 1987).

Comparison of CMTI-I with CMTI-I* and *CMTI-III**. Panels A and B of Figure 2 compare the chemical shifts of backbone hydrogens of CMTI-I* and CMTI-I. Data for CMTI-I correspond to the condition of 25 °C and pH 4.3, as obtained by Holak et al. (1989a). A temperature dependence study of CMTI-I* in the range 10–45 °C (data not shown) indicates that the chemical shifts of $C_\alpha H$'s do not change at all, whereas those of peptide NH's change within a range of 0.04 ppm for 5 °C. Thus, the temperature variation between two data sets causes negligible or no change in the chemical shift positions of a few NH's. Thus, peptide NH's of Arg5,

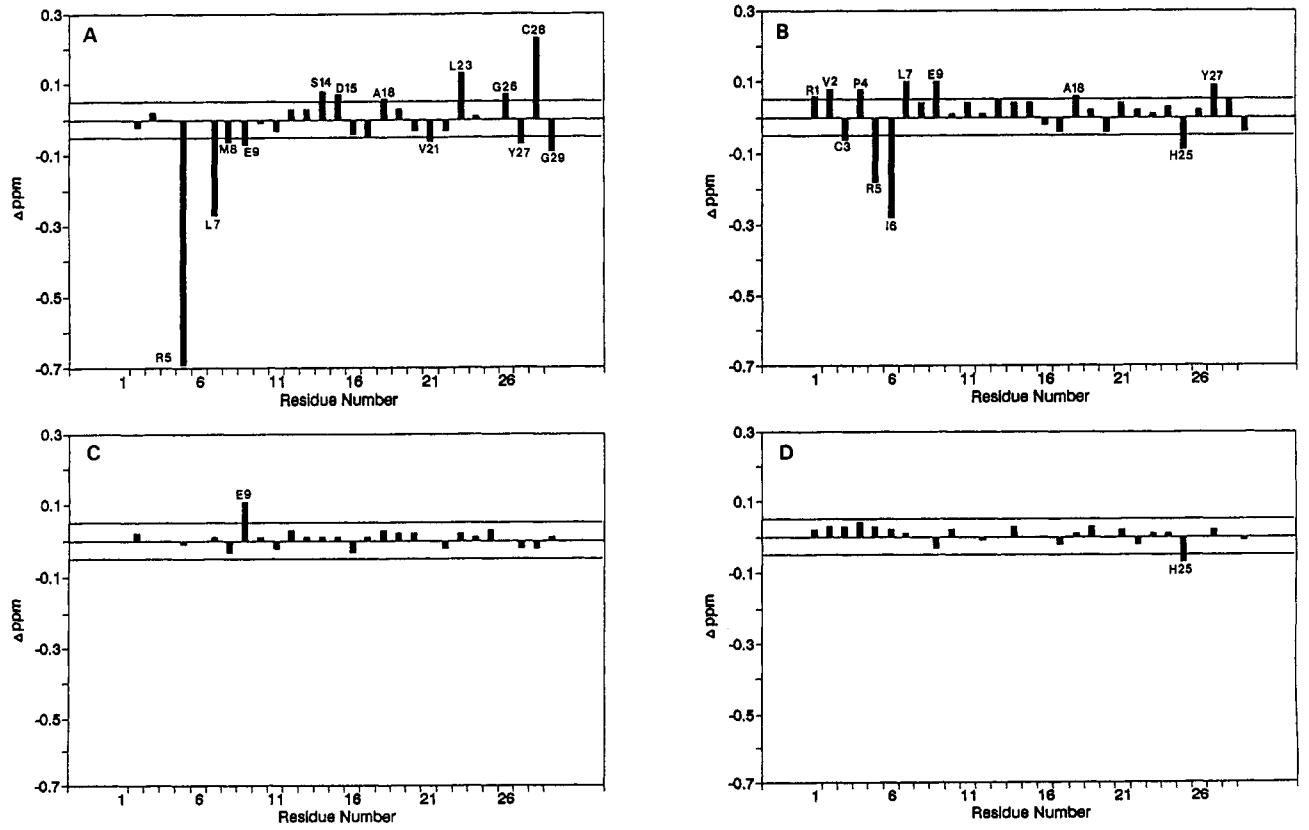

FIGURE 2: Differences in chemical shifts of backbone hydrogens between modified (CMTI-I*) and intact (CMTI-I) squash inhibitor and between CMTI-I* and CMTI-III* has a Lys in position 9, whereas CMTI-I* has a Glu (Wieczorek et al., 1985). Chemical shift data for CMTI-I are taken from the work of Holak et al. (1989a). Chemical shift data for CMTI-III* are taken from the work of Krishnamoorthi et al. (1992a). (A) NH chemical shift difference (CMTI-I* – CMTI-I); (B) $C_\alpha H$ chemical shift difference (CMTI-I* – CMTI-I); (C) NH chemical shift difference (CMTI-I* – CMTI-III*); (D) $C_\alpha H$ chemical shift difference (CMTI-I* – CMTI-III*). The Arg5–Ile6 peptide bond is cleaved in CMTI-I* and CMTI-III*. For Gly26 and Gly29, only one of the two $C_\alpha H$ peaks (downfield shifted) is represented. The horizontal lines enclosing the range ±0.05 ppm represent the experimental error.

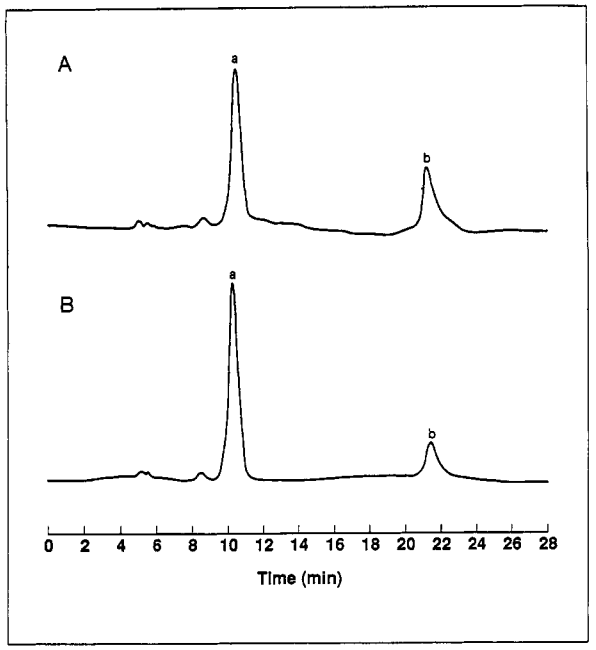

FIGURE 3: HPLC traces of an equilibrium mixture of intact (CMTI-III) and modified (CMTI-III*) inhibitors at two different temperatures: (A) 55 °C; (B) 2 °C. Peak a belongs to CMTI-III* and peak b belongs to CMTI-III, as established by amino acid sequence analysis (Krishnamoorthi et al., 1990).

Leu7, Met8, Glu9, Ser14, Asp15, Ala18, Val21, Leu23, Gly26, Tyr27, Cys28, and Gly29 all show significant changes, with the largest change of about −0.7 ppm observed for Arg5 (Figure 2A). Similarly, the $C_\alpha H$'s of Arg1, Val2, Cys3, Pro4, Arg5, Ile6, Leu7, Glu9, Ala18, His25, and Tyr27 show significant changes (Figure 2B). Similar effects were earlier noted in a comparative study of CMTI-III and CMTI-III* (Krishnamoorthi et al., 1992a). Thus, cleavage of the reactive-site peptide bond leads to tertiary structural changes throughout most of the inhibitor molecule. Panels C and D of Figure 2 compare the chemical shifts of backbone NH and $C_\alpha H$ atoms of CMTI-I* and CMTI-III*, respectively: except for residues Glu9 and His25, none of the residues is affected by the E9K substitution. CMTI-III* has Lys9, whereas CMTI-I* has Glu9. It is of interest to note that the pK_a of His25 is 5.72 ± 0.02 in CMTI-I and 5.97 ± 0.02 in CMTI-I* (Krishnamoorthi et al., 1992b). The HOHAHA contour map of a mixture of CMTI-I* and CMTI-III* (data not shown) revealed doubling of NH peaks only for residues Met8, His25, and Gly26. On the other hand, a comparison of the intact proteins, CMTI-I and CMTI-III, revealed that the chemical shifts of backbone hydrogens of residues Val2, Pro4, Arg5, Met8, Lys9, Asp15, Cys16, Leu17, Ala18, Glu19, Cys20, Cys22, His25, Gly26, and Gly29 are affected by −0.15 to 0.09 ppm (Krishnamoorthi et al., 1992a). Most of these amino acid residues make up the secondary structural elements of the inhibitor. However, the same E9K substitution has no or negligible effect on the backbone hydrogens of these residues, once the protein is modified. In order to gain an insight into the disappearance of perturbation in chemical shifts due to the amino acid substitution, E9K, in the modified forms of the inhibitors, we proceeded to investigate the equilibrium between the intact and modified inhibitors.

Thermodynamics of the Equilibrium between Intact and Modified Inhibitors. Figure 3 shows the HPLC traces of the equilibrium mixture at two selected temperatures, 2 and 55 °C. As can be seen, the relative proportion of CMTI-III* decreases with increasing temperature. The K_{hyd} values obtained at different temperatures were plotted as a function of $1/T$ (Figure 4) and fitted to a straight line corresponding to the van't Hoff equation. The slope and intercept corresponding to the straight line yield, respectively, the following thermodynamic parameters: $\Delta H°$ = −3.16 ± 0.69 kcal/mol and $\Delta S°$ = −8.89 ± 0.37 eu. In the absence of specific information about solvent–polypeptide interactions for the intact and modified inhibitor, a rigorous interpretation of thermodynamic parameters is not possible. The results suggest increased stabilization of the reactive-site-hydrolyzed inhibitor relative to the intact form in water and likely ordering of solvent water molecules caused by the cleavage of the Arg5–Ile6 peptide bond. Such ordering of solvent molecules has been known to decrease the entropy of systems involving ionization of organic acids in water (March, 1977). The $\Delta G°$ for the equilibrium, intact inhibitor ⇌ modified inhibitor, at 30 °C was calculated to be −607 ± 18 cal/mol for CMTI-III and −669 ± 19 cal/mol for CMTI-I.

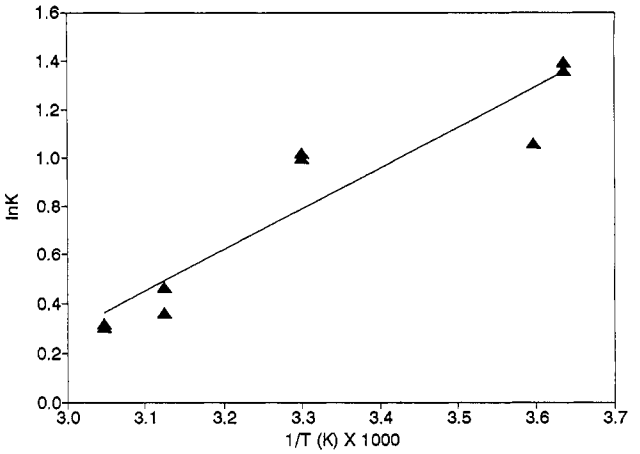

FIGURE 4: Temperature dependence of the equilibrium, CMTI-III ⇌ CMTI-III*, where K = [CMTI-III*]/[CMTI-III]. The data points were fitted to a straight line corresponding to the van't Hoff equation, and the following thermodynamic parameters were obtained: $\Delta H°$ = −3.16 ± 0.69 kcal/mol; $\Delta S°$ = −8.89 ± 0.37 eu.

Conclusions. Structural perturbations caused by the naturally occurring amino acid substitution, E9K, on the intact forms of pumpkin seed trypsin inhibitors, CMTI-I (Glu9) and CMTI-III (Lys9), are apparently removed when the reactive-site peptide bond between Arg5 and Ile6 is hydrolyzed. Thus, the hydrogen chemical shifts of backbone atoms of CMTI-I* and CMTI-III* are not affected, although small differences in chemical shifts have been observed for residues making up the secondary structural elements in the intact native proteins. In each case, the reactive-site-hydrolyzed inhibitor is more stable than the intact inhibitor, with $\Delta G°$ being about −600 cal/mol at 30 °C.

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SUPPLEMENTARY MATERIAL AVAILABLE

Sequential assignments and a complete listing of observed NOE cross peaks of CMTI-I* (5 pages). Ordering information is given on any current masthead page.

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Substrate Specificities of Tissue Kallikrein and T-Kininogenase: Their Possible Role in Kininogen Processing†

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ABSTRACT: The present studies demonstrate the importance of subsite interactions in determining the cleavage specificities of kallikrein gene family proteinases. The effect of substrate amino acid residues in positions P_3–P'_3 on the catalytic efficiency of tissue kallikreins (rat, pig, and horse) and T-kininogenase was studied using peptidyl-pNA and intramolecularly quenched fluorogenic peptides as substrates. Kinetic analyses show the different effects of D-amino acid residues at P_3, Pro at P'_2, and Arg at either P'_1 or P'_3 on the hydrolysis of substrates by tissue kallikreins from rat and from horse or pig. T-Kininogenase was shown to differ from tissue kallikrein in its interactions at subsites S_2, S'_1, and S'_2. As a result of these differences, Abz-FRSR-EDDnp with Arg at P'_2 is a good substrate for tissue kallikreins from horse, pig, and rat but not for T-kininogenase. Abz-FRRP-EDDnp and Abz-FRAPR-EDDnp with Pro at P'_2 (rat high molecular weight kininogen sequence) are susceptible to rat tissue kallikrein but not to tissue kallikreins from horse and pig. Arg at P'_3 increased the susceptibility of the Arg–Ala bond to rat tissue kallikrein. These data explain the release of bradykinin by rat tissue kallikrein and of kallidin by tissue kallikreins from other animal species. Abz-FRLV-EDDnp and Abz-FRLVR-EDDnp (T-kininogen sequence) are good substrates for T-kininogenase but not for tissue kallikrein. Arg at the leaving group (at either P'_1, P'_2, or P'_3) lowers the K_m values of T-kininogenase while Val at P'_2 increases its k_{cat} values. The results indicate that the enzyme subsites S'_1, S'_2, and S'_3 are important determinants for the substrate specificity of tissue kallikreins and T-kininogenase. The findings are also in agreement with the known species specificity of tissue kallikreins and the resistance of rat T-kininogen to tissue kallikreins.

Tissue kallikreins are a group of serine proteinases encoded by a multigene family which consists of very closely related members (Mason et al., 1983). Recently, a proteinase with T-kininogenase activity, isolated from rat submandibular gland, was also characterized as a member of this group (Xiong et al., 1990). Understanding the mechanism of limited proteolysis of kininogens by kallikreins and related proteinases is of interest for many reasons. These enzymes may play a regulatory role in physiological processes since the kininogen substrates, as well as the released kinin peptide products, display very important physiological functions (Schachter, 1980). Studies on kininogen processing may also provide useful information about other kallikrein gene family members such as tonin (Lazure et al., 1981) and enzymes involved in the

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