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Conformational and Immunochemical Analysis of the Cyanogen Bromide Fragments of Thermolysin†

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ABSTRACT: In order to explore the possible existence of folding domains predicted on the basis of protein morphology, we carried out conformational studies for the three cyanogen bromide fragments of thermolysin containing residues 1–120 (FIII), 121–205 (FI), and 206–316 (FII). As judged by far-ultraviolet circular dichroism, FII retains a relative content of α-helical structure similar to that exhibited by the corresponding region in native protein. The conformation of the fragment exhibits significant thermostability. Fragment FI, for which the corresponding region in the native protein contains all of the residues involved in the binding of both the functional zinc and three of the four calcium ions, exhibits a calcium-induced α-helical structure, again as judged by circular dichroism. In order to relate the spectroscopically defined conformations of fragments FI and FII to those of the corresponding regions in native protein, we prepared antisera in rabbits by using thermolysin, FI, and FII as immunogens. As indicated mainly by immunodiffusion, and for FI gel filtration radioimmunoassay, a significant amount of cross-reactivity exists between each of the fragments, especially FII, and native but not denatured (heat-treated, N^{ϵ}-succinylated) thermolysin. The immunochemical data obtained indicate that both FI and FII have the propensity to form stable conformations in solution that are comparable to those required for antigenicity in native thermolysin. The overall conformational data for fragments FI and FII agree with the view that peptides corresponding to domains in globular proteins can form native-like structures independently of the remainder of the molecule.

Physicochemical, functional, and immunological properties of fragments of well-characterized proteins often have been used as a means of elucidating the nature of the forces that direct the folding of sequences of amino acids into specific, biologically active conformations. Fragments obtained from various proteins generally have been found to contain less secondary structure than they exhibit in corresponding regions of intact proteins (Crumpton & Small, 1967; Epand & Scheraga, 1968; Scatturin et al., 1967; Hermans & Puett, 1971; Toniolo et al., 1975). Hence, it is likely that there is a critical set of information required to obtain the necessary medium- and long-range interactions that stabilize the local

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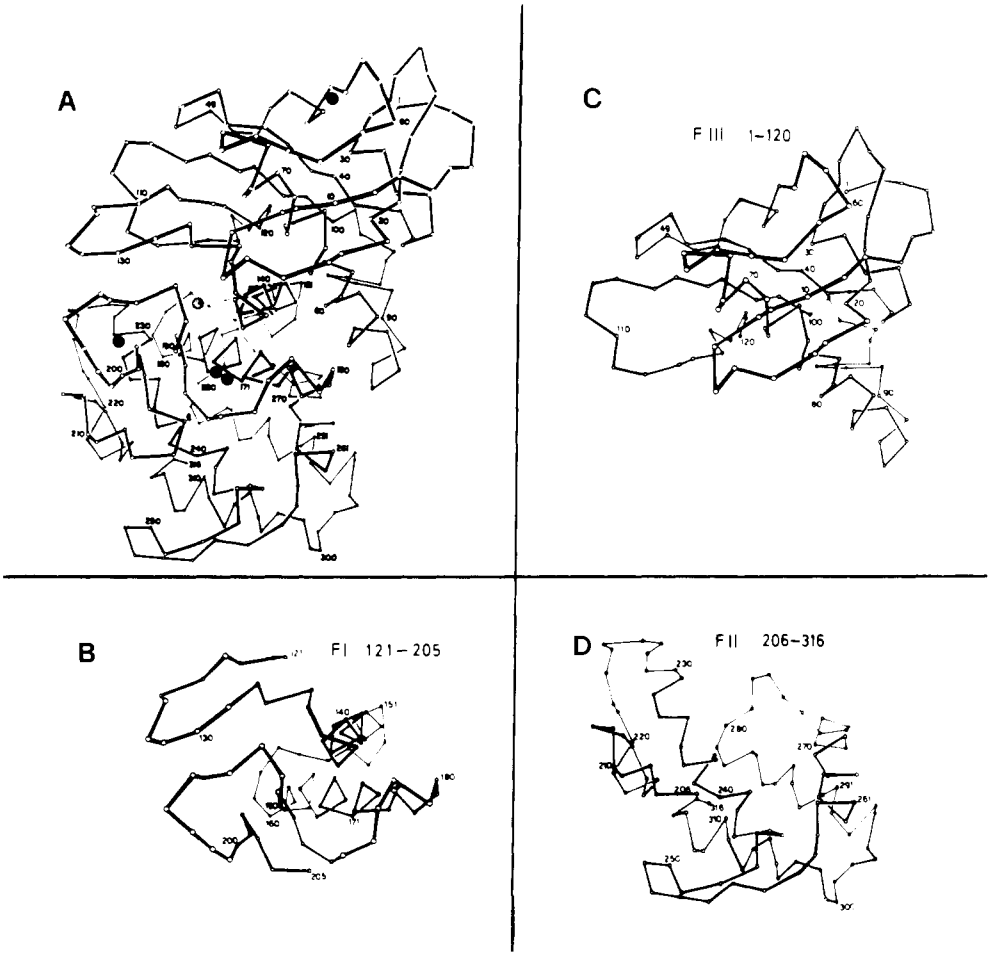

FIGURE 1: (A) Backbone diagram of the thermolysin crystallographic structure showing the single zinc ion binding site (○) and the four calcium ion binding sites (●). (B–D) Backbone diagrams of the CNBr fragments of thermolysin as the corresponding regions exist in native thermolysin. Taken with permission from Colman et al. (1972).

conformations which result from short-range interactions (Wetlaufer & Ristow, 1973; Anfinsen & Scheraga, 1975). It has been proposed that virtually the entire polypeptide chain of a protein is required to provide the information for folding into a native conformation (Taniuchi & Anfinsen, 1968, 1969; Taniuchi, 1970).

In spite of the general requirements of overall sequence, evidence has been obtained in some cases that isolated protein fragments have the innate propensity to attain native-like three-dimensional structures. Substantial amounts of α helix have been found in two central, long peptides from myoglobin, the amount of helix being dependent on the solvent composition (Singhal & Atassi, 1970; Atassi & Singhal, 1970). Several fragments of bovine serum albumin have been shown to retain some conformational features similar to those of the corresponding regions of the parent protein (Reed et al., 1975). More strikingly, the re-formation of native-like structures by reduced fragments of albumin was shown by immunochemical criteria (Teale & Benjamin, 1976a,b, 1977). Two fragments of the β subunit of tryptophan synthetase showed independent folding (Högberg-Raibaud & Goldberg, 1977a,b). More recently, fragment 13–105 of hen egg-white lysozyme was shown by ligand binding and immunochemical data to regenerate native-like structure (Johnson et al., 1978). These various observations have been interpreted by considering that single chain protein structures of more than 150 amino acids could be composed of independently folded regions, denoted domains. Wetlaufer (1973) proposed that these domains might be viewed as intramolecular subunits capable of self-assembly, so that fragments corresponding to a continuous chain region would be prone to maintain the structural features of that segment in the intact protein. With respect to protein folding, domains also might represent independent nucleation and growth regions (Wetlaufer, 1973; Teale & Benjamin, 1976a,b, 1977; Johnson et al., 1976, 1978; Karplus & Weaver, 1976).

The present work was undertaken to evaluate the possibility of semiautonomous domain folding in thermolysin, a thermostable endoprotease (Endo, 1962; Ohta, 1967) for which the amino acid sequence (Titani et al., 1972) and three-dimensional structure (Matthews et al., 1972, 1974; Colman et al., 1972) have been elucidated. This single polypeptide chain of 316 amino acid residues can be cleaved at the two methionyl residues at positions 120 and 205 with CNBr to produce three protein fragments of comparable size (Titani et al., 1972), namely, FIII (1–120), FI (121–205), and FII (206–316).[1] Based on the bilobal structural morphology of thermolysin (Figure 1A) and the distribution of fragments in the sequence (Figure 1B–D), it was predicted that the CNBr pieces might retain some of the structural features of the corresponding regions in the parent thermolysin molecule. In particular, the

[1] Abbreviations used: CD, circular dichroism; FI, FII, and FIII, the fragments containing respectively residues 121–205, 206–316, and 1–120 of thermolysin; anti-thermolysin, anti-FI, and anti-FII, antibody preparations obtained by using as antigen thermolysin, FI, and FII, respectively; denatured thermolysin, heat-treated N^{ϵ}-succinylated thermolysin; RIA, radioimmunoassay; PBS, phosphate–saline buffer (0.075 M NaCl-0.017 M KH_2PO_4–0.057 M $NaHPO_4$, pH 7.4); Tris, tris(hydroxymethyl)-aminomethane; CNBr, cyanogen bromide.

COOH-terminal fragment FII, corresponding to approximately two-thirds of one of the two lobes of native thermolysin, might be expected to form an independently folded structure if the domain hypothesis were operative here. Similarly, FI contains all of the amino acid residues, as defined by X-ray crystallography (Matthews et al., 1972, 1974; Colman et al., 1972), which bind both the functional zinc ion and three of the four calcium ions and thus might be expected to exhibit ion-binding properties. The present report describes both a spectroscopic analysis of the secondary structures of the thermolysin fragments by using far-ultraviolet circular dichroism (CD) measurements and an immunochemical characterization of the relationship between fragment conformation and that of native protein.

Experimental Procedures

Materials. Thermolysin from *Bacillus thermoproteolyticus* (Rokko) was obtained from Calbiochem (San Diego, CA) as a lyophilized product containing 40% calcium acetate. The enzyme was purified by affinity chromatography (Pangburn et al., 1976; Walsh et al., 1974) by using an affinity matrix of glycyl-D-phenylalanine covalently attached to aminoalkyl-Sepharose. To preserve active enzyme, we stored purified protein at −20 °C in 20 mM Tris-HCl buffer, pH 7.2, containing 10 mM $CaCl_2$. For fragmentation, the active enzyme eluted from the affinity column was denatured by precipitation by adding trichloroacetic acid to a final concentration of 1% in order to prevent autolytic degradation, separated by centrifugation, and then used for fragmentation with CNBr. Amino acid analysis, electrophoresis on cellulose polyacetate, and specific activity (Pangburn et al., 1976) established the homogeneity of the purified, native thermolysin. Crystalline bovine serum albumin was obtained from Armour Pharmaceutical Co. (Kankekee, IL) and used without further purification. CNBr was a Fluka (Buchs, Switzerland) product. Urea (C. Erba, Milan, Italy) was recrystallized twice from 95% ethanol and used only in fresh solutions. Tris was from Fluka, calcium chloride from C. Erba, and $K^{14}CNO$ from Amersham Radiochemicals and cellulose polyacetate strips (4 × 12 cm, Cellogel) were from Chemetron (Milan, Italy) and Sephadex G-25, G-50SF, G-75SF, and G-100 from Pharmacia (Uppsala, Sweden). Other reagents used were of analytical grade.

Amino Acid Analysis. Peptide and protein samples were hydrolyzed with 6 N HCl in evacuated, sealed tubes at 110 °C for 20 h. Samples were analyzed with a JEOL amino acid analyzer, Model JLC-6AH, by using the single-column procedure. No corrections were made for destruction or slow release during hydrolysis.

Electrophoresis. Analytical electrophoresis on cellulose polyacetate strips was carried out in 0.04 M sodium glycinate buffer, pH 9.5, containing 6 M urea, at 150 V for 2 h. The strips were stained with 2% Amido-Schwartz in methanol–water–acetic acid (45:45:10) for 15 min and then destained by washing with the same solvent mixture but of different composition (95:95:10).

Preparation of CNBr Fragments. The CNBr cleavage of thermolysin was carried out by using a procedure similar to that described by Titani et al. (1972). In a typical experiment, 50 mg of CNBr was added to a solution of 100 mg of purified, trichloroacetic acid inactivated thermolysin in 10 mL of 70% formic acid. The mixture was stirred in the dark at room temperature for 20 h and, after dilution with 50 mL of water, evaporated in vacuo at 37 °C and then freeze-dried. The residue was redissolved in a minimum volume of a mixture of acetic acid–formic acid–water (40:10:50) and applied to a column (3.3 × 140 cm) of Sephadex G-50SF equilibrated with the same solvent mixture. The column was eluted at a flow rate of 20 mL/h, and fractions of 6 mL were collected and monitored for absorbance at 280 nm. The fractions corresponding to three peaks of peptide material, almost completely separated, were pooled and lyophilized. The first peak was shown to correspond to fragment 1–205 by amino acid analysis, the second to a mixture of FIII and FII, and the third to FI.

The separation of FIII and FII was achieved by dissolving their mixture (second Sephadex G-50SF fraction) in 0.1 M ammonium bicarbonate, pH 8.0, containing 8 M urea, and then diluting with 4 volumes of water. Upon addition of water, precipitation of FIII occurred, whereas FII and some FIII remained in solution. The precipitation procedure was repeated once. The supernatant solutions were combined, concentrated by evaporation in vacuo, and passed through Sephadex G-75SF (3.3 × 135 cm) equilibrated with 5% formic acid. Fractions of 5 mL were collected at a flow rate of 20 mL/h and analyzed at 280 nm. Two well-separated peaks were obtained, the first corresponding to FII and the second to FIII. The fractions were pooled and lyophilized. The homogeneity of the CNBr fragments thus obtained was confirmed by electrophoresis on cellulose polyacetate and amino acid analysis.

Radioisotopic Labeling of FI. The peptide (0.6 mg, 66 μmol), dissolved in 400 μL of 0.2 M sodium phosphate buffer, pH 8.5, and 400 μL of dimethyl sulfoxide, was added to $K^{14}CNO$ (0.4 μmol, 20 μCi) in 0.65 mL of phosphate buffer. After 15 h at room temperature, the reaction mixture was diluted with 100 μL of 99% formic acid. Labeled FI was purified by gel filtration on Sephadex G-25 (1 × 45 cm) eluted with 5% formic acid at a flow rate of 15 mL/h. Fractions (3.2 mL) were analyzed at 280 nm and assayed for radioactivity. [*carbamoyl*-^{14}C]FI, eluted as the first radioactive peak, was stored in 20 mM Tris-HCl, pH 7.2, containing 10 mM $CaCl_2$, at −20 °C until use.

Preparation of Denatured Thermolysin. To a stirred solution of thermolysin (50 mg, 1.4 μmol) in 3 mL of water containing 5 mg of EDTA brought to pH 8.0 with solid $NaHCO_3$ was slowly added finely ground succinic anhydride (300 mg, 3 mmol) over a 15-min period at room temperature. The pH of the reaction mixture was maintained at 8.0 by adding solid $NaHCO_3$. After being stirred for 2 h, the solution was applied to Sephadex G-75SF (2.1 × 104 cm) equilibrated with 20 mM Tris-HCl buffer, pH 7.2, with elution at a flow rate of 5 mL/h. Fractions (3.5 mL) were monitored for absorbance at 280 nm, with those corresponding to the protein peak pooled and stored at −20 °C.

An aliquot of the N^{ϵ}-succinylated thermolysin in 20 mM Tris-HCl, pH 7.2, was heated at 80 °C for 5 min. This sample of heat-treated, N^{ϵ}-succinylated thermolysin showed a far-ultraviolet circular dichroism spectrum indicative of largely random structure (A. Fontana, unpublished experiments) and is used in the present study as a denatured form of thermolysin, possessing insignificant amounts of periodic secondary structure.

The extent of N^{ϵ}-succinylation was estimated by reaction of the modified protein with excess 1-fluoro-2,4-dinitrobenzene in $NaHCO_3$ solution, pH 8.2 (Sanger, 1949), and subsequent acid hydrolysis with 6 N HCl and automatic amino acid analysis. Free ε-amino groups, if present, would be arylated by 1-fluoro-2,4-dinitrobenzene, such that lysine would not be regenerated upon acid hydrolysis. Since the recovery of lysine was in agreement with that expected for thermolysin (Titani et al., 1972), it can be concluded that complete N^{ϵ}-succiny-

lation of lysine residues occurred.

Production of Antisera. New Zealand white female rabbits were immunized, one each with thermolysin (0.9 or 0.25 mg/mL), FI (0.3 mg/mL), or FII (0.6 mg/mL). Each immunogen (0.5 mL) was added to 0.5 mL of complete Freund's adjuvant. The immunization was administered at multiple sites intradermally. After 1 week and thereafter for 3 weeks, boosts were given with incomplete Freund's adjuvant and the same dose of immunogen. Animals were bled 3 days after the second boost and weekly thereafter for up to 3 months.

Immunodiffusion Assay. Rabbit antisera were analyzed by Ouchterlony double-immunodiffusion analyses (Ouchterlony, 1948) on "Immunoplate" pattern C (Hyland Division, Travenol Laboratories, Inc., Costa Mesa, CA). For routine analyses, 7.5 μL of antiserum was placed in the center well and allowed to diffuse against 7.5 μL of antigen solution [thermolysin (0.9 mg/mL), FI (0.3 mg/mL), or FII (0.3 mg/mL), each dissolved in PBS buffer]. Plates were examined for up to 30 h for precipitin lines. The latter usually were observed after 4–8 h if they formed at all. Competition experiments usually were performed in which a sample (7.5 μL) containing equal volumes of competing antigen solution and antiserum was diffused against a second sample (7.5 μL) containing equal volumes of antigen solution and PBS buffer. In such assays, failure of a precipitin band to form or formation of a significantly weaker band was considered to be evidence for antibody recognition of the competing antigen.

Radioimmunoassays. Radioimmunoassay of binding of FI to thermolysin antibodies was carried out by the Sephadex G-100 gel filtration method essentially as described before (Fischer et al., 1977). Antiserum (25 μL) was incubated for 30 min at ambient temperature with 0.06 mg of [*carbamoyl*-^{14}C]FI in 75 μL of PBS containing 0.01% sodium azide as a preservative and 20 μL of 10% bovine serum albumin in PBS. The mixture then was eluted on a Sephadex G-100 column (1 × 23 cm) with PBS containing 1% bovine serum albumin. In the competition assays the above incubation was carried out in the presence of 0.18 mg of either thermolysin or denatured (heat-treated, N$^{\epsilon}$-succinylated) thermolysin.

Protein and Peptide Fragment Concentration. Protein and fragment concentrations were routinely determined spectrophotometrically. The concentration of thermolysin was determined from absorbance determinations at 280 nm by using $A_{280nm}^{1\%}$ = 15.2 (Voordouw & Roche, 1974). Concentrations of fragments were determined by using $A_{280nm}^{1\%}$ = 22.8 for FIII, 13.3 for FI, and 8.6 for FII. The $A_{280nm}^{1\%}$ values for the fragments were calculated on the basis of their known amino acid compositions (Titani et al., 1972) and by using a molar extinction coefficient at 280 nm of 5690 and 1280 for tryptophan and tyrosine, respectively (Edelhoch, 1967). The concentrations determined spectrophotometrically compared favorably (±3%) with those based on quantitative amino acid analysis. When necessary, protein or fragment solutions were clarified by passing them through a Millipore filter (0.45 μm).

Circular dichroism (CD) measurements were performed with a Cary 61 automatic recording dichrograph equipped with a thermostatically controlled cuvette holder through which water was circulated with a Haake bath. The temperature inside the cell was measured with an immersed thermistor probe. The mean residue ellipticity values, [θ], are expressed in deg cm^2 dmol^{-1} and were calculated by the equation $[\theta] = (\theta/10)(MRW/lc)$, where θ is the observed ellipticity, l is the path length in cm, and c is the protein or peptide fragment concentration in g/mL. MRW is the mean residue molecular weight, calculated on the basis of the known amino acid compositions (Titani et al., 1972) and taken as follows: thermolysin, 108.6; FI, 106.7; FII, 106.5; FIII, 111.9. Measurements were made by using cylindrical fused quartz cells of a path length of 0.05 or 0.1 cm. The CD intensity was calibrated by using an aqueous solution of *d*-10-camphorsulfonic acid (Cassim & Yang, 1969). Dynode voltage never exceeded 600 V. Spectra were routinely corrected for base-line shifts by running samples of solvent buffer.

Table I: Amino Acid Composition of Thermolysin and Its Cyanogen Bromide Fragments[a]

amino acids	thermolysin	FIII (1–120)	FI (121–205)	FII (206–316)
aspartic acid	42.0 (44)	19.8 (21)	11.5 (12)	10.6 (11)
threonine	24.7 (25)	10.5 (11)	5.6 (6)	8.0 (8)
serine	25.2 (26)	9.5 (9)	5.2 (6)	10.6 (11)
glutamic acid	19.2 (21)	5.6 (5)	7.8 (8)	8.7 (8)
proline	8.8 (8)	2.2 (2)	3.2 (3)	3.1 (3)
glycine	36.3 (36)	11.7 (12)	11.4 (11)	13.1 (13)
alanine	28.6 (28)	11.3 (11)	5.9 (6)	10.6 (11)
valine	19.6 (22)	6.7 (7)	5.0 (6)	8.0 (9)
methionine	1.7 (2)			
isoleucine	16.5 (18)	4.0 (4)	7.0 (8)	5.0 (6)
leucine	15.9 (16)	5.1 (5)	4.5 (5)	6.2 (6)
tyrosine	27.3 (28)	13.8 (15)	4.7 (5)	7.2 (8)
phenylalanine	9.7 (10)	3.9 (4)	2.9 (3)	3.1 (3)
lysine	11.3 (11)	3.0 (3)	1.1 (1)	6.8 (7)
histidine	7.6 (8)	2.7 (3)	1.9 (2)	2.8 (3)
arginine	9.9 (10)	4.9 (5)	1.2 (1)	4.0 (4)

[a] Compositions are expressed as moles of amino acid residues per mole of protein or peptide fragment. Numbers in parentheses indicate expected integral values calculated from the sequence of thermolysin determined by Titani et al. (1972). The values reported indicate the results of analyses carried out in duplicate and are not corrected for losses upon hydrolysis.

The fractional composition for each secondary structure present in thermolysin and CNBr fragments was calculated by using the method for curve fitting to the experimental CD data according to Chen et al. (1972, 1974). The method assumes that the far-ultraviolet CD spectrum may be described as a linear combination of contributions of α-helix, β-pleated sheet, and random forms. The mean residue ellipticity, [θ], would then be the sum of the contributions from each form expressed as the product of the fraction, f, of the molecule in that form and the ellipticity if the polypeptide were totally in that form. The fractions (f_α, etc.) of α, β, and random (R) structures were calculated by using the following relationships: $[\theta] = f_\alpha[\theta]_\alpha + f_\beta[\theta]_\beta + f_R[\theta]_R$ and $f_\alpha + f_\beta + f_R = 1$. Values for $[\theta]_\alpha$, $[\theta]_\beta$, and $[\theta]_R$ were those reported by Chen et al. (1974).

Other Methods. Absorbance values at single wavelengths were measured with a Hitachi Perkin-Elmer Model 139 spectrophotometer, while continuous absorption spectra were obtained with a Cary 15 double-beam spectrophotometer. Fluorescence measurements were carried out at 25 °C with a Hitachi Perkin-Elmer Model MPF-2A spectrofluorometer, equipped with a thermostated cell holder and connected to a Hitachi QPD$_{33}$ recorder. Measurements of pH were made with a Metrohm Model E-510 pH meter equipped with a combined glass electrode.

Results

Characterization of Thermolysin Fragments. The CNBr fragments prepared as described in Experimental Procedures were characterized by amino acid analysis. As shown in Table I, amino acid analyses of these fragments compare favorably with the compositions derived from the sequence of thermolysin

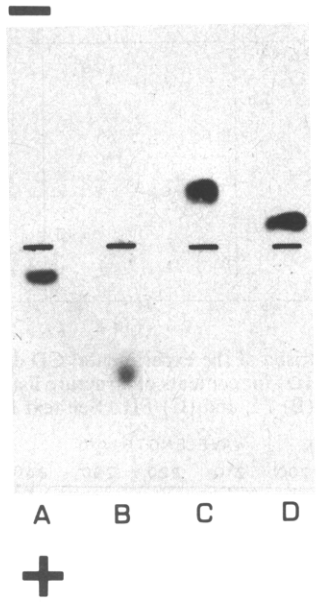

FIGURE 2: Electrophoresis on cellulose acetate strips (Cellogel) (4 × 12 cm) of thermolysin and its CNBr fragments: (A) thermolysin; (B) FI; (C) FII; (D) FIII.

(Titani et al., 1972). As expected (Gross, 1967), homoserine was found in the acid hydrolysates of FIII and FI, but not in that of the C-terminal FII. The homogeneity of the CNBr fragments was also confirmed by analytical electrophoresis on cellulose acetate in the presence of 6 M urea at pH 9.5. All fragments gave single, rather sharp bands (Figure 2). The separation of the fragments obtained by the present method appears superior to that achieved by the $NaDodSO_4$ disc gel electrophoresis method previously used (Titani et al., 1972).

To complete the characterization of the fragments, we recorded their absorption and fluorescence emission spectra. Absorption spectra (not shown) of the fragments dissolved in 5% formic acid showed maxima of absorption at 275–280 nm, as would be expected for polypeptides containing tyrosine and tryptophan. A shoulder at 290 nm, typical of tryptophan residues, was evident in the spectrum of FIII and FI, but not, as expected, in that of FII. By use of an excitation wavelength of 290 nm, FIII and FI showed fluorescence emission spectra typical of tryptophan-containing polypeptides, with maxima, in both cases, near 350 nm (Brand & Witholt, 1967). Fragment FII showed only tyrosine fluorescence emission, with a maximum near 303 nm, and no peaks or shoulders near 350 nm. These results are in agreement with the amino acid composition of the fragments and, in particular, confirm the purity of FII, which has eight tyrosyl residues per molecule and no tryptophanyl residues. Fluorometric analysis of FII was routinely used to assess the purity of the isolated peptide material.

During the course of this study, we found fragment FIII to be considerably insoluble under many conditions. This situation has limited the extent of studies carried out for this fragment in the present work.

Far-Ultraviolet CD Studies. The ultraviolet CD spectral characteristics between 190 and 250 nm, a region associated with secondary structural features of polypeptides (Greenfield & Fasman, 1969; Sears & Beychok, 1973; Chen et al., 1972, 1974), were studied for the CNBr fragments (Figure 3). Since fragment FIII was insoluble at neutral pH, it could be studied meaningfully only at pH 8.5. As shown in Figure 3A, this peptide shows negative ellipticity from 250 to 210 nm. A faint shoulder occurs near 220 nm, with an ellipticity, $[\theta]$, of

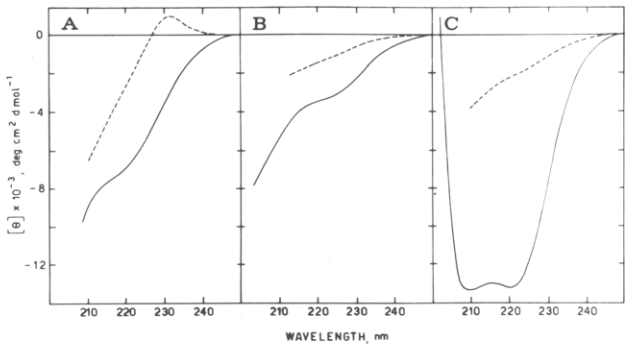

FIGURE 3: CD in the far-ultraviolet region of CNBr fragments of thermolysin. (A) 0.3 mg/mL FIII in 20 mM Tris-HCl buffer, pH 8.2 (—), and in 8 M urea in the same buffer, pH 7.2 (---); (B) 0.2 mg/mL FI in 20 mM Tris-HCl, pH 7.2 (—), and in 8 M urea in the same buffer (---); (C) 0.2 mg/mL FII in 20 mM Tris-HCl buffer, pH 7.2 (—), and in 8 M urea in the same buffer (---).

-7000 ± 300. These characteristics would indicate that FIII has no extensive conformational order. Fragment FI in Tris buffer, pH 7.2, also seems largely disordered, showing again only a small shoulder near 220 nm with ellipticity, $[\theta]$, of -4000 ± 300 (Figure 3B). In contrast, FII in Tris buffer, pH 7.2, possesses a substantial amount of α-helical structure (Figure 3C) since its far-ultraviolet CD spectrum is characterized by the two typical troughs in the region of 210 and 222 nm with ellipticity, $[\theta]$, of $-13\,000 \pm 300$.

The far-ultraviolet CD spectra of CNBr fragments in the presence of 8 M urea are shown by the broken curves in Figure 3. High concentrations of urea are known to disrupt the conformation of many polypeptides and proteins (Tanford, 1968; Wong & Tanford, 1973). The CD spectra of the CNBr fragments in 8 M urea are indicative of polypeptides in an extensively disordered conformation (Nozaki et al., 1974; Leach et al., 1975; Cortijo et al., 1973). Since the CD spectra of FI and FIII are sensitive to perturbation by urea, the residual structures shown by these fragments in aqueous solutions may not be negligible. However, from the differences in spectra obtained in denaturing vs. nondenaturing solutions, it would seem that only FII maintains substantial periodic structure in aqueous solution.

Since FI contains all of the amino acid residues (mostly aspartic and glutamic acid) involved in binding the functional zinc ion and three of the four calcium ions in the native thermolysin (Colman et al., 1972), the effect of calcium on the far-ultraviolet CD characteristics was studied. As shown in Figure 4A, addition of Ca^{2+} ions to a solution of the fragment in Tris buffer, pH 7.2, causes a marked increase in negative ellipticity in the 200–250-nm region, the CD spectrum thus obtained in 0.2–0.9 M $CaCl_2$ being typical of a polypeptide with a substantial portion of the peptide bonds in an α-helical conformation, i.e., with minima near 208 and 222 nm. As indicated in Figure 4B, for $[\theta]$ at 220 nm vs. $CaCl_2$ concentration, the ion-induced transition in secondary structure is rather sharp between 0 and 0.05 M $CaCl_2$ and linear in the 0.1–0.9 M $CaCl_2$ range.

Analysis of Secondary Structure. The fraction of α-helix, β-pleated sheet, and nonperiodic conformation of proteins can be calculated if one assumes additivity of their optical activity (Saxena & Wetlaufer, 1971; Chen et al., 1972, 1974). Taking the ellipticity values of each form determined for proteins of known X-ray structure (Chen et al., 1974), it was possible to calculate their contributions in FI (in 0.9 M $CaCl_2$) and FII. In order to analyze the content of secondary structure in native thermolysin, we recorded the far-ultraviolet CD spectrum of the enzyme in 20 mM Tris-HCl buffer, pH 7.2, containing

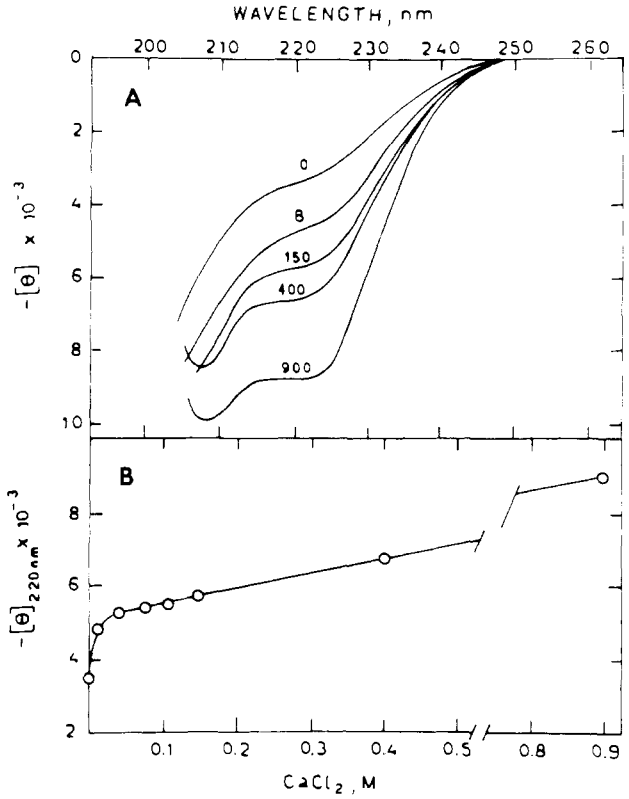

FIGURE 4: (A) Effect of Ca^{2+} ion concentration on the far-ultraviolet CD spectrum of FI dissolved (0.2 mg/mL) in 20 mM Tris-HCl buffer, pH 7.2. Numbers near the curves indicate millimolar concentrations of $CaCl_2$. (B) Effect of increasing concentrations of $CaCl_2$ on the mean residue ellipticity, $[\theta]$, at 220 nm of FI in Tris buffer, pH 7.2.

Table II: Secondary Structures of Thermolysin and Its CNBr Fragments FI (121–205) and FII (206–316) as Determined by Circular Dichroism[a]

protein or fragment	f_α	f_β	f_R
thermolysin[b]	0.29 (0.34)	0.30 (0.20)	0.41 (0.46)
FI (121–205)[c]	0.30 (0.40)	0.11 (0)	0.59 (0.60)
FII (206–316)[d]	0.45 (0.49)	0.05 (0)	0.50 (0.51)

[a] The fractions (f_α, f_β, and f_R) of α, β, and random structures were computed by using the method of Chen et al. (1974). Values of $[\theta]$ were those proposed for helical segments of an average length of eight residues. The far-ultraviolet CD spectra of thermolysin and its CNBr fragments were all recorded at room temperature in 20 mM Tris-HCl buffer, pH 7.2. In parentheses, values are given of the fractional amounts of α, β, and random conformations predicted from the X-ray crystallographic structure of thermolysin (Matthews et al., 1974). [b] Protein at a concentration of 0.106 mg/mL in Tris buffer containing 10 mM $CaCl_2$. [c] Peptide FI at a concentration of 0.2 mg/mL in Tris buffer containing 0.9 M $CaCl_2$. [d] Peptide FII at a concentration of 0.2 mg/mL in Tris buffer with no added $CaCl_2$.

10 mM $CaCl_2$. The CD spectrum of thermolysin shows characteristics typical of helical polypeptides, with $[\theta]_{210nm}$ −11 200 and $[\theta]_{222nm}$ −8900. The numerical fittings obtained with the parameters of Chen et al. (1974) are illustrated in Figure 5. The fractions of the various conformations so calculated are listed in Table II, along with those for native thermolysin.

Thermal Stability of FI and FII. In view of the thermostability of the parent thermophilic enzyme (Ohta, 1967; Pangburn et al., 1976; Fontana et al., 1977), the effects of heat on the conformations of the thermolysin fragments were studied. As shown in Figure 6, the far-ultraviolet ellipticity of FI in 20 mM Tris buffer, pH 7.2, containing 0.18 M $CaCl_2$, undergoes a distinct temperature-induced unfolding (decrease

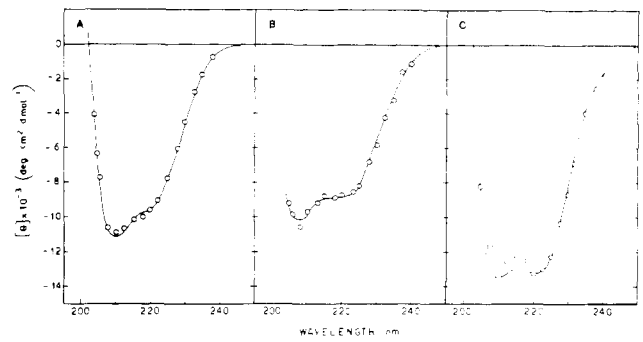

FIGURE 5: Comparison of the experimental CD data (—) with the computed spectra (O) for contents of structure listed in Table II for (A) thermolysin, (B) FI, and (C) FII. See text for details.

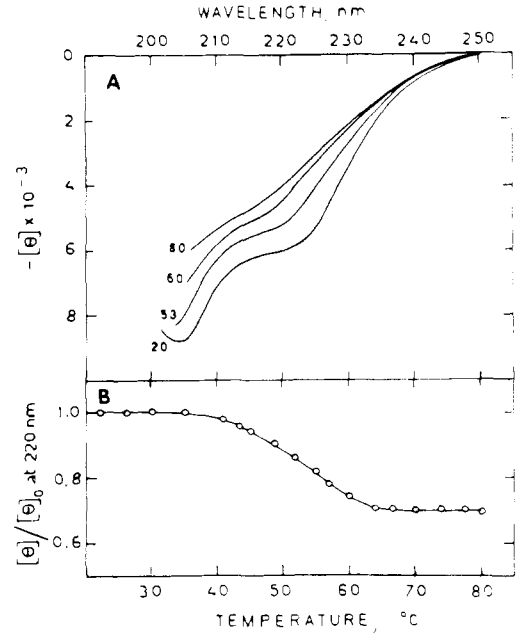

FIGURE 6: (A) Temperature dependence of the far-ultraviolet CD of 0.2 mg/mL FI in 20 mM Tris-HCl buffer, pH 7.2, containing 0.18 M $CaCl_2$. Numbers near the curves indicate the temperatures at which the spectra were recorded. (B) Effect of increasing temperature on the mean residue ellipticity at 220 nm of 0.2 mg/mL FI in Tris-HCl buffer, pH 7.2, containing 0.18 M $CaCl_2$. The results are reported as $[\theta]/[\theta]_0$ vs. temperature, where $[\theta]_0$ is the ellipticity value at 22 °C.

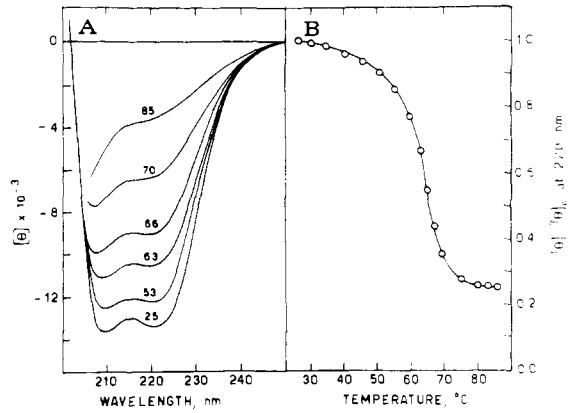

FIGURE 7: (A) Far-ultraviolet CD spectra of 0.2 mg/mL FII at different temperatures in 0.1 M Tris-HCl buffer, pH 7.2. Numbers near the curves indicate temperatures. (B) Ellipticity at 220 nm of FII as a function of temperature, reported as $[\theta]/[\theta]_0$ vs. temperature, where $[\theta]_0$ is the ellipticity value at 25 °C.

in negative ellipticity in the 210–230-nm region), with a broad but cooperative transition in the range of 40–60 °C. Similar

Table III: Summary of Immunological Results with Antisera to Thermolysin and Its CNBr Fragments FI (121–205) and FII (206–316)

immunogen	antigen	immunodiffusion response[a]	competing antigen	competition response[b]
thermolysin	thermolysin	+	thermolysin	+
	denatured thermolysin	very weak +		
	FI[c]	–		
	FII	+	thermolysin	+
			denatured thermolysin	very weak +
			FII	+
FI (121–205)	thermolysin	+	thermolysin	+
			denatured thermolysin	very weak +
			FI	weak +
	denatured thermolysin	very weak +		
	FI	weak +	thermolysin	+
			denatured thermolysin	very weak +
			FI	+
FII (206–316)	thermolysin	–		
	denatured thermolysin	–		
	FII	+	thermolysin	+
			denatured thermolysin	very weak +
			FII	+

[a] Responses: (+) precipitin lines appear; (–) no precipitin lines appear; (weak +) faint lines appear; (very weak +) trace, almost negligible lines appear. [b] Responses: (+) precipitin lines disappear; (–) lines unchanged; (weak +) lines diminished; (very weak +) trace, almost negligible change in lines. [c] Radioimmunoassays indicate binding interaction of FI with anti-thermolysin (Figure 9).

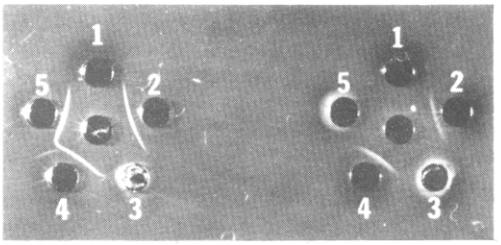

FIGURE 8: Immunodiffusion assays of anti-thermolysin interactions with native thermolysin and FII. All wells contained 7.5-μL total volume of species as defined below. (Left) Center well, 0.45 mg/mL thermolysin; 1, prebleed serum diluted 2:1 with PBS; 2, anti-thermolysin serum diluted 2:1 with PBS; 3, 2:1 diluted anti-thermolysin serum containing 0.45 mg/mL thermolysin; 4, 2:1 diluted anti-thermolysin serum containing 0.45 mg/mL denatured thermolysin; 5, 2:1 diluted anti-thermolysin serum containing 0.15 mg/mL FII. (Right) Same as left except that center well contains 0.15 mg/mL FII.

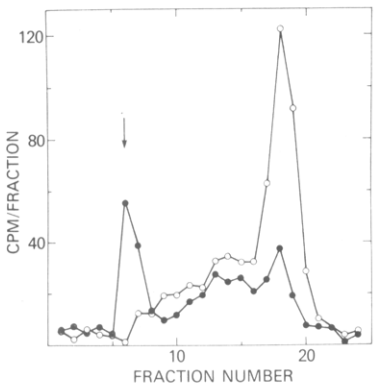

FIGURE 9: Gel filtration radioimmunoassay of anti-thermolysin interaction with FI. Details are provided under Experimental Procedures. (●) [*carbamoyl*-^{14}C]FI + anti-thermolysin serum; (○) [*carbamoyl*-^{14}C]FI + anti-thermolysin serum + thermolysin. The solid arrow indicates the peak position of antibody-bound [*carbamoyl*-^{14}C]FI. Fractions contained 20 drops, or 1.2 mL. Elutions carried out (but not shown, for simplicity) with [*carbamoyl*-^{14}C]FI + either (a) anti-thermolysin serum + denatured thermolysin or (b) anti-FI serum both show profiles similar to that for [*carbamoyl*-^{14}C]FI + anti-thermolysin serum. Assays with the prebleed serum from the anti-thermolysin rabbit show no bound FI peak.

results were obtained at higher concentrations (up to 0.9 M) of Ca^{2+} ions. Upon cooling, the thermal transition was not fully reversible.

Figure 7A shows the family of far-ultraviolet CD curves observed with FII in Tris buffer, pH 7.2, on going from 25 to 85 °C. The prominent features of the helix-related CD spectrum disappear, and at 85 °C the spectrum observed is consistent with that of a largely unfolded polypeptide. Figure 7B shows the temperature dependence of ellipticity, $[\theta]$, normalized at 25 °C, with a sharp, cooperative thermal transition in the range of 60–70 °C. A melting temperature (T_m) of 65 ± 1 °C was measured in three different experiments. The thermal transition of the fragment as followed by CD measurements was shown to be fully reversible.

Immunochemical Properties of Thermolysin Fragments. In order to assess the relationship between fragment conformation and the conformation of the corresponding regions in intact thermolysin, we studied the fragments with respect to their antigenic as well as immunogenic properties. Antisera to thermolysin were obtained in rabbits and tested for specific interaction with both proteins and fragments. As indicated in Figures 8 and 9 and Table III, anti-thermolysin sera can recognize not only thermolysin itself (as assessed by immunodiffusion) but also FII (by immunodiffusion) and FI (by radioimmunoassay). In all cases, these responses are suppressed in competition assays when the homologous antigen species is mixed in the antiserum well at the onset of immunodiffusion or when this species is mixed with antiserum and [*carbamoyl*-^{14}C]FI in the incubation before gel filtration radioimmunoassay. On the other hand, direct immunodiffusion assays of anti-thermolysin with denatured (heat-denatured, N^{ϵ}-succinylated) thermolysin, as well as competition assays with this species, all show at most only weak interactions of anti-thermolysin with this species. Thus, FI and FII are each recognized by anti-thermolysin antibodies which differentiate between native and disordered protein, therein demonstrating the presence of antigenic sites in the fragments that are also present in native but not denatured protein.

Fragments FI and FII also exhibit thermolysin-specific immunogenicity. As indicated in Table III, these two fragments elicit antisera which not only form precipitates with the fragments against which they are elicited but also recognize native (but not denatured) thermolysin (for anti-FI, seen by direct precipitation; for anti-FII, seen by competition of precipitation of FII). The precipitates, when formed, are

suppressed by the appropriate competing species in immunodiffusion. Thus, FI and FII each contain antibody-eliciting determinants specific for intact, native thermolysin.

Discussion

The FI and FII cyanogen bromide fragments of thermolysin, each representing about one-third of the 35 000 molecular weight protein, both show spectroscopic characteristics indicative of a tendency to form organized conformations in aqueous solution. For FII, this structure is largely α helical as judged by CD data. Furthermore, this helical structure, which corresponds to the predominant type of ordered conformation that this region attains in the native protein (Figure 1), is considerably stable as judged by a T_m estimated at about 65 °C (Figure 6).

Fragment FI also shows a considerable, albeit Ca^{2+}-dependent, tendency to form a stable conformation in aqueous solution. Although CD data suggest little ordered structure when alone, addition of Ca^{2+} leads to significant helicity (Figure 4). Although the Ca^{2+} requirement for this transition is high, the effect can be correlated with the known calcium stabilization of thermolysin (Endo, 1962; Feder et al., 1971) as well as the existence of calcium-binding residues in the FI region of the native protein as judged by X-ray crystallographic data (Matthews et al., 1972, 1974). Like the conformation of FII, that for Ca^{2+}-stabilized FI is rather resistant to heat, with a T_m of about 50 °C (Figure 5). Examination of the FI sequence reveals that at neutral pH there would be 14 evenly distributed carboxylate groups of aspartic and glutamic acid residues along the polypeptide chain of 85 amino acids. Repulsive interactions between the negatively charged carboxylates might be expected to interfere with helix formation. Binding of Ca^{2+} ions to the polypeptide chain could provide a means of allowing formation of intrachain hydrogen bonds required for the helical structure.

In order to relate the regular structure observed in FI and FII with that of the corresponding fragments within the intact native thermolysin, we obtained global estimates of secondary structure on the basis of mean residue ellipticity values in the far-ultraviolet region. It must be kept in mind that fitting CD spectra of polypeptides with constructs based on fixed mixtures of α-helix, β-pleated sheet, and unordered forms remains problematical (Baker & Isemberg, 1976; Garnier et al., 1976; White, 1976). Nonetheless, we have estimated the extent of α, β, and random forms both in thermolysin and in fragments FI and FII by using the method of Chen et al. (1974) (Table II). With thermolysin, the calculations employed appear to underestimate the α-helix content and overestimate the β-sheet content. On consideration of the limitations of the methods employed, the data of Table II suggest that the fragments attain amounts of secondary structure in solution comparable to those observed in the corresponding sequences in the native enzyme.

Although the above spectroscopic data do not directly relate the stable conformations of FI and FII in aqueous media with the actual detailed conformations of the corresponding regions in the native protein, the immunochemical properties of these fragments do address this issue. That these fragments contain immunogenic and antigenic sites similar to those in the native protein is clear from results with both anti-thermolysin and anti-fragment sera. Anti-thermolysin sera form precipitates not only with native thermolysin but also with FII. While precipitation is not observed with thermolysin antisera vs. FI, the recognition of this fragment by anti-thermolysin is apparent from radioimmunoassay with [*carbamoyl*-^{14}C]FI. In addition, both anti-FI and anti-FII sera recognize thermolysin as indicated in immunodiffusion assays. All of the above antibody interactions were shown to be specific by the lack of significant interactions with denatured (heat-treated, N^{ϵ}-succinylated) thermolysin and by the lack of response with appropriate preimmune sera, as well as by successful competition with appropriate intact protein or fragment species. Given the relative nonantigenicity of denatured thermolysin as well as the general view that protein antigenic determinants are conformational in many cases, the immunochemical data in Table II support the contention that FI and FII both form native-like conformational features in solution. The relative equilibria governing the formation of these conformational entities cannot be deduced from the present data, due largely to the lack of pure antibodies. Nonetheless, the results described here strongly suggest that at least some elements of the conformations of FI and FII apparent in their spectroscopic characteristics are related to the native conformations of the corresponding regions in intact thermolysin.

The experimental evidence reported here for the conformational integrity of thermolysin fragments is consistent with previous data describing the conformational propensities of other protein fragments (see introductory part of this paper). To be sure, fragments which have been found to form recognizable conformations in aqueous solution in general do so only to a small extent at equilibrium. Thus, as shown for staphylococcal nuclease fragments (Taniuchi & Anfinsen, 1968), the vast majority of molecules at any given time are disordered, such that spectroscopic measurements generally reveal little overall structure. However, methods which allow detection of relatively small percentages of ordered species within larger populations of disordered species, such as the use of specific antibodies directed against native conformations, have allowed the demonstration of small populations of folded species (Sachs et al., 1972). Indeed, the immunochemical approach used for fragments such as those of nuclease has here given strong evidence of the conformational propensities of FI and FII.

On consideration of all of the data obtained here, FI and FII appear to represent a case of fragments, predicted to have specific conformational propensities based on protein morphology, for which the structures indeed appear to be substantially stable in aqueous media as judged by their spectroscopic characteristics. This is especially true for FII, but also for FI when stabilized by Ca^{2+} (which presumably acts by binding known Ca^{2+}-binding residues in the 121–205 region). Thus, these two fragments not only have the propensity to form native-like local conformational interactions but also apparently form enough stabilizing interactions to preserve these structures at equilibrium. That FI and FII exhibit such behavior may be related to their being large species corresponding to discrete domains in the intact protein, i.e., a Ca^{2+}-binding region for FI and an α-helical lobe in the case of FII. In this regard, the data lend support to the concept that individual conformational domains of proteins contain enough sequence information to achieve folded conformations at least semiindependently from the rest of the protein. If, as proposed before (Wetlaufer, 1973), domain folding represents a general step in the pathway(s) of folding of a particular protein, the folding of thermolysin may involve both the independent folding of the carboxyl-terminal end corresponding to FII and the Ca^{2+}-promoted folding of the middle region corresponding to FI.

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