Domain Characteristics of the Carboxyl-Terminal Fragment 206–316 of Thermolysin: Unfolding Thermodynamics†

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ABSTRACT: The carboxyl-terminal fragment 206–316 of thermolysin is a single polypeptide chain without disulfide bridges which has been shown to maintain a folded structure in aqueous solution comparable to that of the corresponding region in native thermolysin [Vita, C., Fontana, A., Seeman, J. R., & Chaiken, I. M. (1979) *Biochemistry 18*, 3023–3031]. The conformational transitions of the fragment induced by heat, guanidine hydrochloride, and urea have been followed with the use of circular dichroism and difference spectroscopy measurements. Thermal denaturation in aqueous solution at pH 7.5 shows a fully reversible and cooperative transition with a midpoint near 66 °C. The energetics of the thermal unfolding process were determined under the two-state assumption by the van't Hoff procedure. The enthalpy of denaturation varied linearly with temperature, and correspondingly, the difference in heat capacity between the folded and thermally unfolded forms of the fragment, ΔC_p, was found to be 1.9 kcal/(mol·deg). A figure of 3.2 kcal/mol for the free energy of stabilization (ΔG) of the fragment in physiological environment at 37 °C was evaluated. The midpoints of the reversible transitions of the fragment at 25 °C induced by guanidine hydrochloride and urea occur at 2.0 and 5.7 M, respectively. The free energy of unfolding of the fragment in the absence of denaturant was found to be 5.6–7.5 kcal/mol, depending upon the denaturant and the method of calculation employed. These results indicate that fragment 206–316 of thermolysin is a polypeptide chain having, under neutral conditions, a stable, folded configuration with thermodynamic properties typical of a small globular protein. The results of this study give experimental support to the prediction that segment 203–316 [Rashin, A. A. (1981) *Nature (London) 291*, 85–87] or segment 212–316 [Wodak, S. J., & Janin, J. (1981) *Biochemistry 20*, 6544–6552] of the thermolysin molecule is a domain (or subdomain) that can be expected to fold by itself into a stable structure.

The structures of large monomeric proteins, as obtained from X-ray diffraction studies, often consist of quite distinct "domains" or compactly folded regions. Such structural domains sometimes are so well separated that some protein molecules appear bi- or multilobal, with the active center of the enzyme located at the interface between domains. Recently, it has become widely accepted that domains are the basic units of protein structure. As a result of this model, it is conceivable to suggest that (i) each of these compact regions can behave as a small protein, so that when excised from the remainder of the polypeptide chain it would be able to fold by itself, and (ii) domains could represent regions of rapid growth of structures during the folding process and that proper interactions between domains would lead to the final conformation of the native protein, in analogy to subunits in oligomeric proteins. This idea of protein folding by a mechanism of modular assembly is of wide contemporary interest and is being tested experimentally in a number of laboratories with fragments of large proteins obtained by limited proteolysis or chemical cleavage [Wetlaufer, 1973; Högberg-Raibaud & Goldberg, 1977a,b; Johnson et al., 1978a; Ghelis et al., 1978; Isenman et al., 1979; Goto & Hamaguchi, 1979; Chavez & Scheraga, 1980; Dautry-Varsat & Garel, 1981; Goldberg & Zetina, 1980; Adams et al., 1980; cf. Wetlaufer (1981) and Rossmann & Argos (1981) for recent and comprehensive reviews].

The term domain has been used to refer to structural domains (as seen by visual inspection of protein models), functional domains (possessing some specific binding or catalytic property), proteolytic domains (as derived by their excision from the rest of the polypeptide chain by limited proteolysis), and folding domains (protein pieces which can independently fold). In order to avoid ambiguities, computer algorithms based on atomic positions have been used to detect domains and subdomains in proteins (Crippen, 1978; Rose, 1979). More recently, surface area measurements (Lee & Richards, 1971) were used by Rashin (1981) and Wodak & Janin (1981) to give a quantitative definition of folding and structural domains, and the results obtained were in good agreement with domains previously identified by folding experiments with protein fragments and by inspection of protein models (Wetlaufer, 1973). Thus, Rashin (1981) defined a domain as a globular fragment corresponding to a segment of a protein chain that, when removed from the rest of the protein, buries a maximum of surface area within its fold, and correspondingly the contribution of the free energy to its stability is maximal. Such a fragment is expected to fold independently from the rest of the protein into a stable structure.

Thermolysin shows a quite peculiar bilobal morphology, with two distinct structural domains of equal size and the active site located at the interface between them (Colman et al., 1972; Matthews et al., 1974). In previous reports (Fontana & Vita, 1977; Vita et al., 1979, 1982), we have evaluated the possibility of domain folding of thermolysin by using fragments obtained by cyanogen bromide cleavage of the protein at the level of the two methionine residues in positions 120 and 205 of the polypeptide chain. In particular, it has been shown that the carboxyl-terminal fragment 206–316 (fragment FII)[1] retains a relative amount of secondary structure similar to that exhibited by the corresponding segment in the native protein (Vita et al., 1979). In addition, antigenic and immunogenic

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[1] Abbreviations: fragment FII, peptide fragment corresponding to sequence 206–316 of thermolysin; CD, circular dichroism; Gdn·HCl, guanidine hydrochloride; Tris, tris(hydroxymethyl)aminomethane.

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properties of the fragment indicated that the structure attained in aqueous solution is nativelike[2] (Vita et al., 1982). Since fragment FII corresponds approximately to two-thirds of the C-terminal lobe of thermolysin, these results suggest that this fragment potentially could be used as a simple and favorable system to study domain properties. The peculiar advantages of fragment FII reside in the fact that it is a simple polypeptide chain of 111 amino acid residues (M_r 11 800) without disulfide bridges or thiol groups and without tightly bound metal ions, cofactors, or prosthetic groups. Therefore, it should serve as an appropriate model for unfolding and refolding studies of an isolated domain, with the ultimate goal being that of attaining a better understanding of the mechanism of folding and in particular of the molecular forces that stabilize its nativelike three-dimensional structure.

This paper deals with the denaturation of fragment FII mediated by temperature, Gdn·HCl, and urea, as followed by far-ultraviolet CD and difference spectroscopy measurements. It was found that the fragment unfolds with a high degree of cooperativity, as often observed with small globular proteins. By use of the two-state model, the results on reversible denaturation have been quantitatively analyzed in order to define the energetics of the unfolding processes. This work represents one of the few studies that have been conducted on the unfolding thermodynamics of relatively small protein fragments bearing domainlike characteristics.

Materials and Methods

Materials. Fragment FII was obtained by cyanogen bromide cleavage of thermolysin, and its purity was assessed by electrophoresis and amino acid analysis, as previously described (Vita et al., 1979). The concentrations of the fragment solutions were determined from absorbance determinations at 280 nm by using an extinction coefficient $A^{1\%}_{280nm} = 8.6$, based on the absorption of the tyrosine residues of the fragment (Vita et al., 1979).

Ultrapure guanidine hydrochloride (Gdn·HCl) was obtained from Pierce Chemical Co. (Rockford, IL). Urea (C. Erba, Milan, Italy) was recrystallized twice from 95% ethanol. Only freshly prepared solutions of urea were employed.

Spectroscopic Measurements. CD measurements were made on a Cary 61 dichrograph equipped with a thermostated cell holder, according to procedures outlined previously (Vita et al., 1979). Ultraviolet absorption spectra were obtained with a Cary 15 double-beam recording spectrophotometer equipped with a 0–0.1 absorbance scale and a jacketed cell compartment.

Denaturation Studies. The experimental details of the spectroscopic measurements used to follow the denaturation of fragment FII induced by heat, Gdn·HCl, and urea are reported in the supplementary material (see paragraph at end of paper regarding supplementary material).

Thermodynamic Analysis. The data of the denaturation transitions of fragment FII induced by heat or denaturant (Gdn·HCl or urea) were treated with the assumption of a two-state reversible process, where the native state (N) is thought to be in equilibrium with the denatured state (D), without any other intermediate species present (Tanford, 1968; Pace, 1975). The transition N $\rightleftarrows$ D can be characterized by the variable f_D, defined as the fraction of the molecule in the denatured state. At any point of the transition curve, f_D can be measured experimentally by eq 1, where y_N, y_D, and y are

$$f_D = \frac{y - y_N}{y_D - y_N} \quad (1)$$

the spectroscopic variables characteristic of the initial, final, and intermediate states, respectively. Since each experimental value along the transition yields a unique value for f_D, the equilibrium constant K_D of the N $\rightleftarrows$ D equilibrium can be derived as shown in eq 2. Only experimental values for f_D

$$K_D = \frac{f_D}{1 - f_D} = \frac{y - y_N}{y_D - y} \quad (2)$$

between 0.1 and 0.9 were considered to give reliable results for K_D. The Gibbs free-energy change from the native to the denatured state is given by eq 3.

$$\Delta G_D = -RT \ln K_D \quad (3)$$

The data of the reversible thermal denaturation were represented in a van't Hoff plot in which $\ln K_D$ is plotted as a function of the reciprocal of the absolute temperature. The intersection of the curve of $\ln K_D$ vs. $1/T$ with the line representing $\ln K_D = 0$ provides a value for T_m (melting temperature). The slope of the van't Hoff plot was related to the standard enthalpy change, ΔH, of the unfolding reaction. Changes in heat capacity, ΔC_p, associated with thermal denaturation of fragment FII were obtained from the slope of ΔH vs. temperature. Thermodynamic parameters characterizing differences between native and thermally denatured states of fragment FII were determined on the basis of eq 4–6,

$$\Delta G = \Delta H_m(1 - T/T_m) - \Delta C_p[(T_m - T) + T \ln (T/T_m)] \quad (4)$$

$$\Delta H = \Delta H_m + \Delta C_p(T - T_m) \quad (5)$$

$$\Delta S = \Delta S_m + \Delta C_p \ln T/T_m \quad (6)$$

which derive from the assumption of a two-state transition and constancy of ΔC_p (Privalov & Khechinashvili, 1974; Tall et al., 1976; Elwell & Schellman, 1977; Schellman & Hawkes, 1980).

The transition curves observed for fragment FII with urea and Gdn·HCl as denaturants were analyzed by eq 1–3 to obtain K_D and ΔG_D values as a function of the denaturant concentration. In order to obtain an estimate of the Gibbs free-energy change from the native to the denatured state in aqueous solution and in the absence of denaturant, $\Delta G_D^{H_2O}$, the data for ΔG_D were plotted according to eq 7, assuming that

$$\Delta G_D = \Delta G_D^{H_2O} - m[D] \quad (7)$$

a linear dependence of ΔG_D on denaturant concentration continues to zero denaturant concentration (Pace, 1975), where [D] is the concentration of urea or Gdn·HCl and m is a coefficient that reflects the steepness of the experimental transition curve, i.e., the degree of cooperativity for the denaturation process.

In a different approach, $\Delta G_D^{H_2O}$ values were obtained on the basis of the side-chain transfer model (Tanford, 1970; Pace, 1975) by eq 8, where ΔG_D is calculated from experimental data

$$\Delta G_D = \Delta G_D^{H_2O} + \alpha \sum_i n_i \delta g_{tr,i} \quad (8)$$

by eq 3, α is the average fractional change of the exposure of amino acid side chains, $\delta g_{tr,i}$ is the free energy of transfer of a particular group of type i from water to aqueous Gdn·HCl or urea calculated from solubility studies on amino acids (Tanford, 1964), and n_i is the total number of groups of type i present in fragment FII calculated from its amino acid composition (Titani et al., 1972; Vita et al., 1979). Owing

[2] The term "native" refers to the conformation attained by fragment FII dissolved in 20 mM Tris-HCl buffer, pH 7.5, containing 0.1 M NaCl at room temperature.

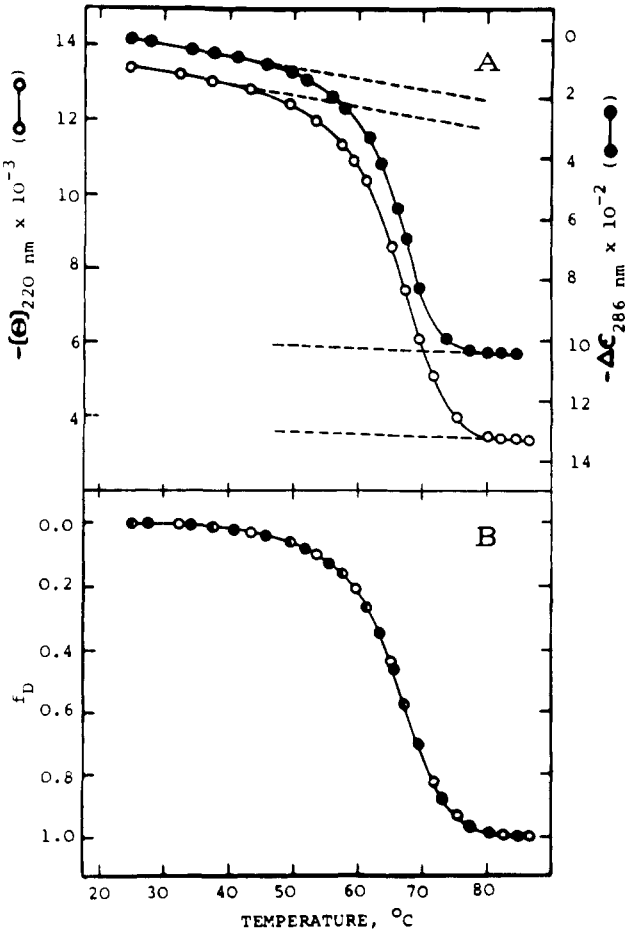

FIGURE 1: Thermal denaturation profiles of fragment FII. (A) (○) Temperature dependence of the mean residue ellipticity, $[\theta]$, at 220 nm of fragment FII dissolved (0.15 mg/mL) in 20 mM Tris-HCl buffer, pH 7.5, containing 0.1 M NaCl. (●) Difference molar extinction, $\Delta\epsilon$, at 286 nm of fragment FII dissolved (0.4 mg/mL) in the same Tris buffer. (B) The fraction of denatured fragment, f_D, is plotted vs. temperature. (○) Mean residue ellipticity, $[\theta]$, at 220 nm; (●) difference molar absorption, $\Delta\epsilon$, at 286 nm.

to the sharpness of the denaturation curves of fragment FII, only one or two values of $\delta g_{tr,i}$, as reported by Pace (1975), fell inside the transition region, and thus values for $\delta g_{tr,i}$ at different concentrations of denaturants were calculated by a parabolic interpolation of the already available values (Tanford, 1970; Pace, 1975).

Results

Thermal Denaturation. Figure 1A shows the temperature dependence of the mean residue ellipticity $[\theta]$ measured at 220 nm, which is a sensitive probe of secondary structure (Greenfield & Fasman, 1969). The spectral changes observed are consistent with a cooperative transition between 55 and 70 °C, with a midpoint near 66 °C. The complete reversibility of thermal unfolding was established by the fact that denaturation curves obtained by raising the temperature were superimposable with those obtained by lowering the temperature.

Because a reversible, two-state unfolding process is required to permit a standard van't Hoff analysis, it was of importance to verify that at low or high temperatures there are only two thermodynamically identifiable states for fragment FII. For assessment of the validity of the two-state assumption, a common criterion used is that transition curves determined by independent experimental parameters are superimposable (Lumry et al., 1966). To this aim, near-ultraviolet CD and difference spectroscopy measurements were carried out, as

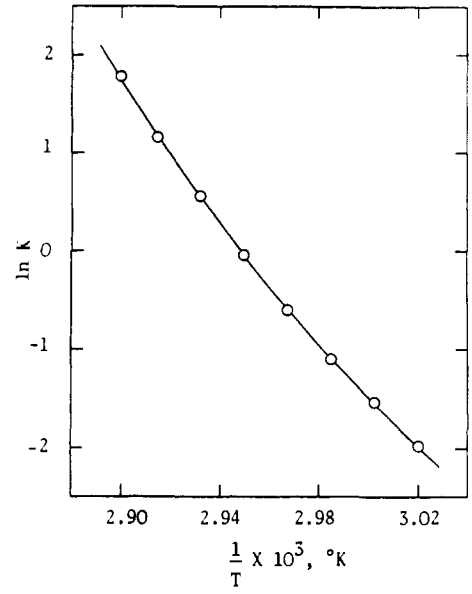

FIGURE 2: van't Hoff plot of the thermal denaturation data for fragment FII. The equilibrium constant (K) for the native-denatured transition was obtained from the data shown in Figure 1. $1/T$ is the reciprocal of the temperature in kelvin.

probes of the environment of the aromatic chromophores.

Initial attempts were done by using the aromatic CD as a probe of the thermal unfolding process of fragment FII. The CD spectrum of the fragment in the 250–300-nm region shows a broad trough of negative ellipticity centered at about 276 nm, with $[\theta]_{276nm} = -100$ deg·cm²·dmol⁻¹ (Figure 1, supplementary material). Since fragment FII lacks tryptophanyl residues or disulfides, the near-ultraviolet CD spectrum is due mainly to tyrosyl residues (Strickland, 1974). However, the aromatic CD did not prove to be a useful experimental parameter to monitor the thermal unfolding of the fragment, since high concentrations (>1 mg/mL) were necessary to obtain a reasonable signal to noise ratio. In addition, changes in ellipticity in the 250–300-nm region due to unfolding were small, allowing few points to be determined with accuracy within the transition region.

The thermal difference absorption spectrum generated on heating from 25 to 85 °C with fragment FII dissolved in 20 mM Tris-HCl buffer, pH 7.5, shows two main negative bands centered near 279 and 286 nm, with a difference molar extinction, $\Delta\epsilon$, of -1050 $M^{-1}\cdot cm^{-1}$ at 286 nm (Figure 2, supplementary material). The temperature dependent change of $\Delta\epsilon_{286nm}$ of fragment FII is shown in Figure 1A. Again, a transition is observed at 55–70 °C, with a midpoint transition near 66 °C. In addition, the curve of $\Delta\epsilon_{286nm}$ vs. temperature was completely reversible.

The transition curves monitored by $[\theta]_{220nm}$ or $\Delta\epsilon_{286nm}$ at any stage of the denaturation process are characterized by the fraction, f_D, of fragment FII in the denatured state, calculated according to eq 1 (see Materials and Methods). In Figure 1B, f_D, calculated from CD or difference absorption data, is plotted vs. temperature. Since it is seen that the two sets of experimental points fall on the same curve, it can be safely assumed that a two-state mechanism is operative in the thermal unfolding process of the fragment.

The results of Figure 1B were used in the calculation of the equilibrium constant K at different temperatures, and the corresponding van't Hoff plot between $-R \ln K$ and $1/T$ is seen in Figure 2. The line obtained shows a slight curvature, as often observed with globular proteins, and is indicative that the ΔH of the unfolding reaction varies with temperature and,

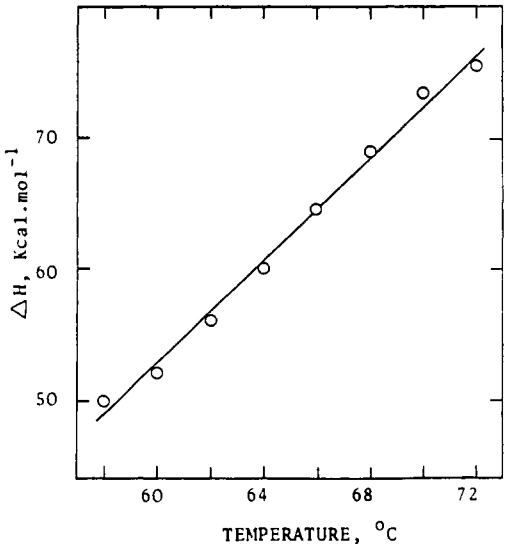

FIGURE 3: Dependence of the enthalpy of denaturation (ΔH) of fragment FII on temperature. ΔH values were obtained from the van't Hoff plot of the spectroscopic data shown in Figure 2. The slope of the line indicates the change in heat capacity associated with denaturation (ΔC_p).

Table I: Thermodynamic Parameters Characterizing the Reversible Thermal Unfolding of the Thermolysin Fragment FII[a]

T_m (°C)	ΔH^b (kcal/mol)	ΔS^b [cal/(deg·mol)]	$\Delta C_p{}^c$ [kcal/(deg·mol)]	ΔG^d (kcal/mol)
66	65	195	1.9	3.2

[a] Calculations were carried out under the assumption of the two-state transition, as described in the text. [b] Determined at T_m. [c] The change in heat capacity that accompanies the unfolding was calculated from the slope of the plot of ΔH, determined by the van't Hoff method, vs. temperature. [d] Calculated at 37 °C, according to eq 4 (see Materials and Methods).

therefore, that the magnitude of the difference in heat capacity, ΔC_p, between the native and unfolded species of fragment FII is different from zero (Brandts, 1969; Tanford, 1970). From the slope of ΔH values obtained from the van't Hoff plot vs. temperature (Figure 3), a figure of 1.9 kcal/(mol·deg) was obtained for ΔC_p. It is relevant to recall that, as expected for a two-state transition (Lumry et al., 1966), the curve describing the dependence of the enthalpy change (ΔH) of denaturation with temperature does not show a maximum near the midpoint of the transition. The parameters ΔH, ΔS, and ΔG describing differences between the native state and denatured state of fragment FII were calculated on the basis of eq 4–6, as reported under Materials and Methods and shown in Figure 3 in the supplementary material. Table I summarizes the thermodynamic parameters characterizing the thermal unfolding reaction of fragment FII.

Guanidine Hydrochloride and Urea Denaturation. The transition of fragment FII from the native to the denatured state induced by Gdn·HCl or urea was followed by measuring CD spectra in the far-ultraviolet region as a function of increasing concentration of denaturant. Upon exposure of fragment FII to a concentration up to 6 M Gdn·HCl or 10 M urea, a gradual decrease in negative ellipticity in the 200–250-nm region was observed (actual CD spectra not shown). The CD spectra in 6 M Gdn·HCl or 10 M urea were indicative of a polypeptide chain in a random conformation (Cortijo et al., 1973). When fragment FII was exposed to 4 M Gdn·HCl in 20 mM Tris-HCl buffer and 0.1 M NaCl, pH

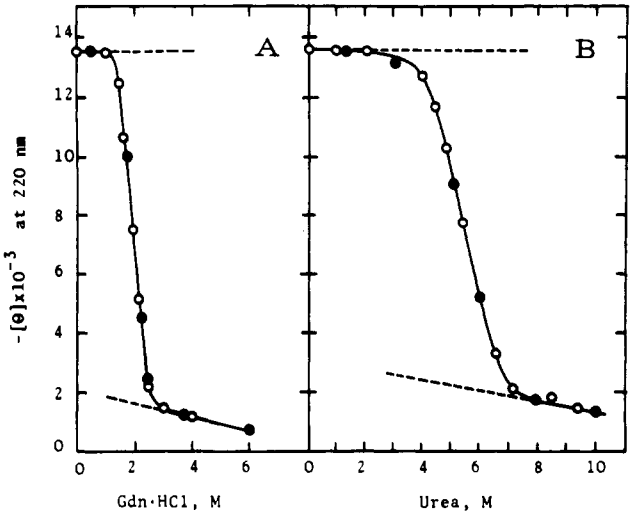

FIGURE 4: Equilibrium curves for the unfolding and refolding of fragment FII in (A) guanidine hydrochloride or (B) urea at 25 °C as followed by circular dichroism measurements. The mean residue ellipticity, $[\theta]$, at 220 nm is plotted as a function of denaturant concentration. The fragment was dissolved (0.15 mg/mL) in 20 mM Tris-HCl buffer and 0.1 M NaCl, pH 7.5, containing guanidine hydrochloride or urea at the indicated molarity. For further details see Materials and Methods. (○) Forward solutions; (●) reverse solutions.

7.5, a negative difference spectrum was generated very much resembling in its features that obtained by thermal unfolding. In this case the difference molar extinction, $\Delta\epsilon$, measured at 286 nm was -1400 $M^{-1}\cdot cm^{-1}$.

The progressive change in the conformation of fragment FII as a function of Gdn·HCl or urea concentration can be seen in terms of the plot of the mean residue ellipticity, $[\theta]$, at 220 nm vs. the denaturant concentration (Figure 4). Fragment FII displays sharp cooperative transitions, as often observed with many globular proteins, with Gdn·HCl being more effective in disrupting the ordered structure of the native state than urea (Tanford, 1968; Pace, 1975). The experimental points obtained from the (direct) denaturation and the (reverse) renaturation experiments lie on the same curve, suggesting that the Gdn·HCl- or urea-mediated unfolding of the fragment is a reversible process. Moreover, the equilibrium was rapidly attained, since no time-dependent effects were seen in the CD spectrometer.

The data of Figure 4 were treated under the two-state assumption according to eq 2 and 3 in order to obtain values for the free energy of unfolding, ΔG_D, inside the transition region (see Materials and Methods). The values of $[\theta]_N$ and $[\theta]_D$, representing the mean residue ellipticities for the native and denatured states of the fragment, respectively, were obtained by extrapolating the linear portions of the denaturation curves into the transition region. As shown in Figure 4, a negative dependence on the concentration of denaturant for $[\theta]_D$ has been observed, while $[\theta]_N$ shows constancy. ΔG_D varies linearly with Gdn·HCl or urea concentration (Figure 5). A least-squares analysis was used to fit the data for ΔG_D to eq 7 (Pace, 1975) in order to calculate $\Delta G_D{}^{H_2O}$ (ΔG_D at zero denaturant concentration) and m (cooperativity index of the transition). These calculated figures are given in Table II.

The variation of ΔG_D with the denaturant concentration can also be described according to the side-chain transfer of amino acids (Tanford, 1970; Greene & Pace, 1974; Pace, 1975) based on the solubilities of amino acid side chains and peptide groups in water and in Gdn·HCl or urea solution. The figures for $\Delta G_D{}^{H_2O}$ and α (degree of exposure of side-chain groups in fragment FII) were calculated according to eq 8 (see Materials

Table II: Parameters Characterizing the Guanidine Hydrochloride and Urea Denaturation of Thermolysin Fragment FII and Ribonuclease[a]

		Gdn·HCl				urea			
compound	method[b]	$D_{1/2}$[c] (M)	m[d] [kcal/(mol·M)]	α[e]	$\Delta G_D^{H_2O}$[f] (kcal/mol)	$D_{1/2}$[c] (M)	m[d] [kcal/(mol·M)]	α[e]	$\Delta G_D^{H_2O}$[f] (kcal/mol)
fragment FII	linear extrapolation[g]	2.0	2.9		5.6	5.7	1.05		5.6
	Tanford's model[h]			0.28	7.5			0.27	6.5
ribonuclease	linear extrapolation[g]	3.0	3.1		9.3	6.9	1.1		7.7
	Tanford's model[h]			0.35	14.8			0.34	12.1

[a] The experimental data for fragment FII are shown in Figures 4 and 5. Calculations of the parameters were carried out as described in the text. The data for ribonuclease are taken from Greene & Pace (1974). [b] cf. Pace (1975), for a review. [c] Denaturant concentration at the midpoint of the transition. [d] The value of m reflects the dependence of the free energy of unfolding on denaturant concentration (see eq 7) (Pace, 1975). [e] The value of α represents the average degree of exposure of side-chain and peptide groups of fragment FII upon unfolding and is calculated by using the side-chain transfer model according to eq 8 (Greene & Pace, 1974; Tanford, 1970; Pace, 1975). [f] Free-energy change in the absence of denaturant. [g] From eq 7. [h] From eq 8.

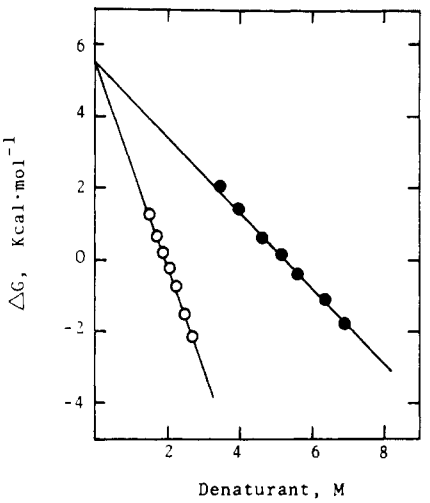

FIGURE 5: Free-energy change, ΔG_D, for the denaturation of fragment FII as a function of (○) guanidine hydrochloride or (●) urea molarity. ΔG_D was calculated by eq 3 as described in the text (Pace, 1975) from the data shown in Figure 4.

and Methods) and are reported in Table II. For the sake of comparison, the parameters describing the Gdn·HCl- and urea-mediated denaturation of bovine pancreatic ribonuclease (Greene & Pace, 1974; Pace, 1975) are also reported in Table II.

Discussion

Denaturation studies of fragment FII by heat, Gdn·HCl, and urea have been carried out in order to give an evaluation of its Gibbs free energy, ΔG, of stabilization under neutral conditions, defined as the free energy required to convert the fragment from its ordered three-dimensional structure to a random-coil polypeptide chain. The data were analyzed under the assumption of a two-state transition, i.e., assuming that there are only two thermodynamically identifiable states (N and D), without intermediate species present in appreciable concentrations at any stage of the denaturation transition. Thus, the two-state model appears largely valid for Gdn·HCl or urea denaturation of a number of globular proteins (Pace, 1975). The agreement between thermal transition curves determined by measuring different physical properties (Figure 1) allows us to conclude that the two-state assumption is a reasonable one (Lumry et al., 1966). This does not imply that by using more refined techniques, e.g., NMR, the existence of metastable folding intermediates could be demonstrated, as already shown with other globular proteins (cf. Baldwin, 1975, for references). The absolute validity of the two-state model for fragment FII is not yet available, and direct evidence for this can be reached by calorimetric analysis (Privalov & Khechinashvili, 1974; Privalov, 1979).

The enthalpy for the thermal unfolding of fragment FII, ΔH, as determined from the van't Hoff plot, varies linearly with temperature, and correspondingly, a positive value is obtained for ΔC_p, the difference in heat capacity between the folded and thermally unfolded forms of the fragment. The parameter ΔC_p is usually correlated with the hydrophobic effect and is explained in terms of the exposure of masked hydrophobic side chains to an aqueous environment upon unfolding, resulting in formation of cages of structured water around these residues, which have both low entropy and high heat capacity (Brandts, 1969; Tanford, 1968, 1970). The figure of 1.9 kcal/(mol·deg) for the ΔC_p of fragment FII is in the range [1–4 kcal/(mol·deg)] previously observed in thermal denaturation studies of several globular proteins (Lumry & Biltonen, 1969; Pace, 1975; Privalov & Khechinashvili, 1974).

The thermodynamic parameters characterizing differences between native and thermally denatured sites of fragment FII were calculated by using eq 4–6 (see Materials and Methods). A plot of ΔG vs. temperature shows a characteristic flattening at low temperatures (see Figure 3, supplementary material), as predicted from two-state thermodynamics with constant ΔC_p (Elwell & Schellman, 1977; Privalov & Khechinashvili, 1974). A figure of 3.2 kcal/mol at 37 °C was obtained, which indicates that the free energy by which the native state of the fragment is stabilized is small, as often observed with globular proteins (Tanford, 1968, 1970; Pace, 1975).

The estimates of the free-energy change of unfolding of fragment FII in the absence of denaturant, $\Delta G_D^{H_2O}$, obtained from Gdn·HCl or urea denaturation are 5.6–7.5 kcal/mol, depending upon the denaturant and the method of extrapolation employed. Pace (1975) discussed on a comparative basis the extrapolation procedures employed to obtain estimates of $\Delta G_D^{H_2O}$. On the basis of the results obtained with several globular proteins, it was possible to conclude that linear extrapolation (eq 7) leads to low estimates of $\Delta G_D^{H_2O}$ and that the Tanford method (eq 8) gives data substantially higher. In addition, it has been found that linear extrapolation, when data from Gdn·HCl or urea denaturation are used, gives value of $\Delta G_D^{H_2O}$ that are in good agreement, as is expected considering the identity of the N $\rightleftarrows$ D reaction. On the other hand, the Tanford method fails to give consistent values for $\Delta G_D^{H_2O}$ using the two denaturants. The figures for $\Delta G_D^{H_2O}$ reported in Table II for fragment FII are in agreement with these general conclusions derived from denaturation studies on a number of globular proteins (Pace, 1975). Therefore, 5.6 kcal/mol should be considered as the lowest estimate of $\Delta G_D^{H_2O}$ for fragment FII.

The figure of ΔG for the Gdn·HCl- or urea-mediated unfolding of fragment FII is few kilocalories per mole greater

than for unfolding to a thermally denatured state. This difference is in line with similar findings obtained with other globular proteins, including ribonuclease (Aune et al., 1967; Tanford, 1968; Tanford & Aune, 1970; Pace, 1975), and has been explained by considering that heat- and Gdn·HCl-unfolded states are not identical. Thus, it was believed that complete unfolding is achieved only in a concentrated Gdn·HCl solution, whereas unfolding due to heat is only partial. However, Privalov (1979) reviewed earlier reports for differences in the extent of protein unfolding depending upon the denaturant employed and concluded that no unequivocal evidence of this fact is available. On the other hand, direct determination of thermodynamic parameters by means of calorimetric measurements indicated that the states of both heat-denatured protein and Gdn·HCl-denatured protein are thermodynamically indistinguishable (Pfeil & Privalov, 1976a–c; cf. Privalov, 1979, Table IV). On the basis of the conclusions reached by Privalov (1979), the figure for ΔG of fragment FII obtained from thermal data can be viewed with caution. Thus, it is worth considering that the calculations of ΔG involved extrapolations over a $\sim$30 °C temperature range and were based on the assumption of constancy of ΔC_p, which is obtained as a second derivative of the original experimental data and cannot be determined accurately.

The slope of the plot of ΔG vs. denaturant concentration gives a figure for m, which measures the dependence of ΔG on denaturant concentration and therefore reflects the ability of a denaturant to unfold a globular protein (Greene & Pace, 1974; Pace, 1975). The values of m observed for fragment FII (Table II) are typical of small globular proteins and remarkably similar to those found with a protein of similar size, i.e., bovine pancreatic ribonuclease (Pace, 1975). As usually found with a number of proteins (Pace, 1975), m(Gdn·HCl) is larger than m(urea) for fragment FII and the ratio m(Gdn·HCl)/m(urea) is 2.76. Thus, this ratio is essentially identical with that found for ribonuclease (2.82) (Greene & Pace, 1974). Considering that the free-energy change associated with the transfer of peptide groups and polar side chains to Gdn·HCl solution are from 2.4 to 2.9 times larger than those observed for the transfer to urea solution, whereas the values for nonpolar side chains are only from 1.6 to 2.3 times greater (Nozaki & Tanford, 1970), it can be proposed that the part of the molecule which unfolds in fragment FII is mainly of polar nature. This conclusion follows the reasoning applied by Greene & Pace (1974) in comparing the Gdn·HCl- and urea-mediated unfolding of ribonuclease and lysozyme.

Several lines of evidence (ΔC_p, α, and near-ultraviolet difference spectroscopy) indicate some masking of hydrophobic side chains in native fragment FII. The figure and significance of α, the degree of exposure of side-chain groups as calculated from Gdn·HCl or urea denaturation studies using Tanford's procedure (eq 8, Table II), is in qualitative agreement with ΔC_p (see above). In fact, the value of α for fragment FII lies in the range (0.20–0.35) already observed with several other proteins (Pace, 1975), in which burial of hydrophobic residues has been extensively documented. The difference absorption spectrum in the 250–300-nm region of fragment FII on heating is negative, and this is usually interpreted, with globular proteins, in terms of a conformational transition in which aromatic chromophores (in this case tyrosine residues) are transferred from a relatively nonpolar environment in the interior of a protein molecule to an aqueous environment (Herskovits, 1967). Considering that a transfer of a single tyrosine residue from the hydrophobic interior of a protein to essentially unhindered contact with solvent would cause a difference molar absorption, $\Delta\epsilon$, at 286 nm of -700 $M^{-1}\cdot cm^{-1}$ (Bigelow, 1961; Donovan, 1973; Herskovits, 1967), heat- or Gdn·HCl-mediated unfolding of fragment FII seems to cause the exposure of additional 1.5–2 out of 8 tyrosine residues of the fragment. On this basis, it can be proposed that hydrophobic interactions resulting from burial of aromatic or other hydrophobic amino acid residues (Kauzmann, 1959) appear to be a contributing factor to the noteworthy stability of the fragment. It is worth mentioning that fragment FII contains a normal percentage of hydrophobic amino acid residues, since the polarity of the fragment, as determined from its amino acid composition (Titani et al., 1972; Vita et al., 1979) by using the method of Capaldi & Vanderkooi (1972), is 47%.

The conformational stability of fragment FII to heat and denaturants is quite high and, as an example, is similar to that observed with ribonuclease, which has a comparable chain length and contains four disulfide bridges (cf. pace, 1975, for references). This is quite remarkable, considering that the additional stabilizing interactions derived from cross-linking and specific binding of ions and cofactors, normally occurring in proteins, are not present in fragment FII. Thus, it is known that intrachain disulfides stabilize significantly the native conformation of many extracellular proteins, which require greater stability to fluctuating environmental conditions [cf. Johnson et al. (1978b) for a discussion on the mechanism of protein stabilization of disulfide bonds].

In summary, the results of this study and of previous ones (Vita et al., 1979, 1982) emphasize that fragment FII of thermolysin is able to fold into a nativelike, stable structure independently from the rest of the protein molecule, with conformational and stability properties that are typical of a small globular protein. In this connection, it is relevant to relate the results herewith described to the calculations recently reported by Rashin (1981) and Wodak & Janin (1981) using algorithms based on surface area criteria to detect the presence of domains within the thermolysin molecule. Rashin (1981) computed two fragments that may be conformationally stable by themselves, the N-terminal segment 4–151 and the C-terminal one 203–316. Wodak & Janin (1981) identified segment 152–316 as a domain containing a globular subdomain 212–316. It is therefore quite interesting to find this close coincidence between the calculated limits of the C-terminal domain (or subdomain) of thermolysin and sequence 206–316 of fragment FII. Thus, fragment FII may be considered almost an ideal model for an isolated protein domain to be used to elucidate on a molecular basis sequence–conformation–stability relationships. A peculiar advantage of this fragment in respect to most of the previously studied protein fragments shown to maintain independent folding (Wetlaufer, 1981) is that the three-dimensional structure of the parent protein, as obtained by X-ray diffraction methods (Colman et al., 1972; Matthews et al., 1974), is known.

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Supplementary Material Available

Experimental details of the spectroscopic measurements used to follow the denaturation of fragment FII induced by heat, Gdn·HCl, and urea. Figures showing the CD spectrum of the fragment in the near-ultraviolet region at pH 7.5, the thermal difference spectrum generated on heating from 25 to 85 °C,

and the temperature dependence of ΔG, ΔH, and ΔS for the thermal unfolding of the fragment (3 pages). Ordering information is given on any current masthead page.

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