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Independent Folding of the Carboxyl-Terminal Fragment 228–316 of Thermolysin†

Claudio Vita, Daniele Dalzoppo, and Angelo Fontana*

Appendix: Prediction of Stabilities of Thermolysin Fragments‡

Alexander A. Rashin

ABSTRACT: The COOH-terminal fragment 206–316 of thermolysin was shown previously to maintain a stable folded structure in aqueous solution comparable to that of the corresponding region in native thermolysin and thus to possess protein domain characteristics [Fontana, A., Vita, C., & Chaiken, I. M. (1983) *Biopolymers 22*, 69–78]. In order to study the effect of polypeptide chain length on folding and stability of an isolated domain, the 111 amino acid residue fragment was shortened on the NH_2-terminal side by removal of a 22-residue segment. Treatment of fragment 206–316 with hydroxylamine under alkaline conditions permitted selective cleavage of the Asn^{227}–Gly^{228} peptide bond, and from the reaction mixture fragment 228–316 was isolated in homogeneous form. This fragment appeared to attain in aqueous solution the folding properties of the corresponding segment in the intact protein, as indicated by quantitative analysis of secondary structure from far-ultraviolet circular dichroism spectra and immunological properties. Thus, double-immunodiffusion analyses showed that fragment 228–316 is able to recognize and precipitate anti-thermolysin antibodies raised in rabbits with native thermolysin as immunogen. The fragment displayed fully reversible and cooperative conformational transitions mediated by pH, heat, and guanidine hydrochloride (Gdn·HCl), as expected for a globular protein species. Thermal denaturation of the fragment in aqueous solution at pH 7.8 showed a T_m of 66 °C and the Gdn·HCl-mediated unfolding a midpoint transition at 2.2 M denaturant concentration. These results correlate well with Rashin's predicted location of stable subdomains in the COOH-terminal portion of thermolysin (see Appendix). An additional interest in the folding and stability properties of fragment 228–316 here described resides in the fact that its nativelike structure is organized as a four-α-helical bundle, a quite recurring protein structural motif [Weber, P. C., & Salemme, F. R. (1980) *Nature (London) 287*, 82–84], herewith shown for the first time to be stable in the absence of disulfide cross-links, bound ions, or cofactors.

Analysis of the three-dimensional structures of monomeric globular proteins has allowed recognition of their specific molecular structural characteristics in terms of a hierarchy of protein substructures, involving domains as one major level of protein structure (Schulz & Schirmer, 1978). Wetlaufer (1973) first surveyed a large set of proteins and by visual inspection identified protein domains as distinct, continuous chain regions that could be enclosed within compact volumes. Later, more quantitative methods to locate structural domains within proteins were developed (Rossmann & Liljas, 1974; Crippen, 1978; Rose, 1979; Sander, 1981; Wodak & Janin, 1981; Rashin, 1981). Some of these approaches (Crippen, 1978; Rose, 1979) allowed a description of protein molecules

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in terms of a hierarchic molecular organization; in particular, domains were shown to be interatively decomposable into subdomains. At the lowest level of this "tree-structural organization of proteins" (Crippen, 1978), one finds continuous segments of protein chain that often coincide well with secondary structure elements (α-helices, β-strands). This hierarchic organization of the native state of a protein has suggested that subdomains and domains lie on the folding pathway of a polypeptide chain from the random state to the final native structure (Wetlaufer, 1973). Thus, it can be proposed that specific segments of a polypeptide chain first refold to individual domains, which then associate and interact to give the final tertiary structure, much the same as do subunits in oligomeric proteins. The major implication from this model is that protein fragments corresponding to domains (or subdomains) should be able to refold by themselves to a nativelike structure. Experimental verification of this assumption has been obtained in a few cases by studying the folding properties of protein fragments obtained by limited proteolysis or chemical cleavage of the parent protein [for review articles, cf. Wetlaufer (1981), Rossmann & Argos (1981), Janin & Wodak (1983), and Ghélis & Yon (1983)].

One relevant aspect in the definition of properties of protein domains is to what extent the entire polypeptide chain of a structural domain is needed to achieve a nativelike and stable structure, i.e., to what extent a fragment can be simplified by deletion of segments or loops without impairing the stereochemistry of its stable folded structure. Rashin (1981) proposed an algorithm for locating polypeptide segments capable of refolding into a stable nativelike structure from the X-ray atomic coordinates of the protein, based on computation of the free energy correlated with the buried surface area (Lee & Richards, 1971). This method allowed the location of stable domains that often coincide with those observed by simple inspection of the protein molecule (Wetlaufer, 1973) and are often contained within them.

We have previously reported the folding properties of the carboxyl-terminal fragment 206–316 (fragment FII)[1] of thermolysin obtained by cyanogen bromide cleavage of the protein at methionine-205 (Vita et al., 1979, 1982; Vita & Fontana, 1982; Fontana et al., 1983). This fragment was shown to be able to refold into a nativelike structure in aqueous solution, as detected by circular dichroism (CD) and immunochemical measurements. This single polypeptide chain of 111 amino acid residues, which lacks thiol or disulfide groups, appears to represent a favorable system for an experimental study of the folding properties of isolated domains. Its peculiar advantage resides in the fact that the three-dimensional structure of the parent protein is known at 1.6-Å resolution (Holmes & Matthews, 1982) and that several theoretical studies on the location of domains in this protein have appeared (Crippen, 1978; Sander, 1981; Rashin, 1981; Wodak & Janin, 1981; Janin & Wodak, 1983). The application of the method of Rashin (1981) to thermolysin (see Appendix) has led to predictions of high probabilities of finding stable nativelike subfragments within fragment FII.

In order to test experimentally the possible existence of nativelike subfragments of the 206–316 piece of thermolysin capable of independent folding, a 22-residue segment has been removed from fragment FII by cleaving the Asn^{227}–Gly^{228} peptide bond with hydroxylamine (Bornstein & Balian, 1977). Structural studies of the isolated fragment 228–316 with CD and immunochemical measurements indicate that this fragment also is able to refold in aqueous solution at neutral pH into a nativelike and stable conformation. The fragment displays cooperative unfolding transitions mediated by pH, heat, and guanidine hydrochloride (Gdn·HCl), as expected for a globular protein species. The experimental results correlate well with the predicted location of stable subfragments within segment 206–316 (see Appendix).

Experimental Procedures

Materials. Thermolysin from *Bacillus thermoproteolyticus* (Rokko) was obtained from Calbiochem (San Diego, CA) and purified by affinity chromatography as previously described (Vita et al., 1979). Fragment FII of thermolysin was obtained by cyanogen bromide cleavage of the protein, and its purity was assessed by electrophoresis and amino acid analysis (Vita et al., 1979).

The materials required for disc gel electrophoresis came from Fluka AG (Basel, Switzerland). Ultrapure guanidine hydrochloride (Gdn·HCl) was obtained from Pierce Chemical Co. (Rockford, IL). Tris, hydroxylamine hydrochloride, and $CaCl_2$ were obtained from C. Erba (Milan, Italy), Sephadex G-50 SF was from Pharmacia (Uppsala, Sweden), diethylaminoethyl-cellulose (DE-52) was from Whatman (Maidstone, Kent, England), and agar was from Riedel-De Haen AG (Hannover, Germany). All other reagents were reagent grade and were used without further purification.

Preparation of Fragment 228–316. The fragment was prepared by NH_2OH cleavage (Bornstein & Balian, 1977) of fragment FII at the level of the Asn^{227}–Gly^{228} peptide bond. This fragment (20 mg) was dissolved in 3 mL of 6 M Gdn·HCl containing 2 M NH_2OH·HCl, pH 9.4. The cleavage reaction was allowed to proceed for 5 h at 45 °C, and then the solution was extensively dialyzed against 1% aqueous formic acid. The solution was then concentrated in vacuo at 37 °C and applied to a Sephadex G-50 SF column (2 × 115 cm) equilibrated with 10% aqueous formic acid. The column was eluted with the same eluent at a flow rate of 11 mL/h, and fractions of 3.2 mL were collected and analyzed spectrophotometrically at 280 nm. A main peak of peptide material was eluted from the column, preceeded by a minor one of unreacted fragment FII. The fractions of the main peak were combined, concentrated in vacuo, and applied again to the same column. A single symmetrical peak was eluted; the corresponding fractions were combined and lyophilized. The isolated fragment 228–316 (8 mg) was analyzed by electrophoresis and amino acid analysis after hydrolysis.

Concentrations of fragment 228–316 were evaluated by using $A_{280nm}^{0.1\%} = 0.65$. This figure was calculated on the basis of its known amino acid composition (Titani et al., 1972) and by using a molar extinction coefficient at 280 nm of 1280 for tyrosine (Edelhoch, 1967). The concentrations determined spectrophotometrically compared favorable (±3%) with those based on quantitative amino acid analysis.

Circular Dichroism. CD measurements were performed with a Cary 61 dichrograph as outlined previously (Vita et al., 1979). The mean residue ellipticity, $[\theta]$, values at the designated wavelength are expressed in deg·cm^2·dmol^{-1} and were calculated by the equation $[\theta] = (\theta/10)/[106.5/(lc)]$, where θ is the observed ellipticity, 106.5 is the mean residue molecular weight, calculated on the basis of the known amino acid composition (Titani et. al., 1972), l is path length in cm, and c is the peptide concentration in g/mL. Estimates of secondary structure from far-ultraviolet CD spectra were obtained by the method of Siegel et al. (1980).

[1] Abbreviations: CD, circular dichroism; fragment FII, thermolysin fragment comprising residues 206–316; Gdn·HCl, guanidine hydrochloride; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

Immunochemical Methods. Antibodies toward native thermolysisn were elicited in rabbits as previously described (Vita et al., 1979). Double immunodiffusion on agar plates was performed by the Ouchterlony technique (Ouchterlony, 1948) with 1% agar in 20 mM Tris-HCl buffer, pH 7.5, containing 10 mM $CaCl_2$. After 1 day at room temperature in a humidified chamber, the agar gel was washed with 5% NaCl solution and then distilled water, dried, and then stained with 1% Coomassie Brilliant Blue R-250 in methanol–acetic acid–water (45:10:45 v/v/v).

Quantitative precipitin reactions were performed in disposable 0.6-mL glass tubes. Increasing amounts of concentrated solution (1.0 mg/mL) of thermolysin, fragment FII, and fragment 228–316, each separately dissolved in 20 mM Tris-HCl buffer, pH 7.5, containing 0.1 M NaCl and 10 mL $CaCl_2$, were added to 90-μL aliquots of the anti-thermolysin serum in a volume of buffer sufficient to bring the total volume of each tube to 0.3 mL. The tubes were vibro mixed and incubated at 25 °C for 30 min and at 4 °C for 20 h. They were then centrifuged, the precipitates washed with ice-chilled saline buffer and dissolved in 0.1 N NaOH, and the absorbances, read at 280 nm, were plotted as a function of the antigen added.

Other Methods. Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis was carried out by using a vertical slab gel apparatus (Laemmli, 1970). Amino acid analyses were performed on a Jeol amino acid analyzer, Model JLC-6AH, after 22 h of hydrolysis in 6 N HCl in evacuated, sealed tubes at 110 °C.

Results

Isolation and Characterization of Fragment 228–316. Fragment 206–316 (fragment FII) of thermolysin contains a single Asn^{227}–Gly^{228} peptide bond (Titani et al., 1972); such a bond should be susceptible to the action of hydroxylamine under mildly alkaline conditions (Bornstein & Balian, 1977). After incubation of the fragment at 45 °C for 5 h with 2 M NH_2OH in 6 M Gdn·HCl, pH 9.4, the reaction mixture was extensively dialyzed (Spectrapor membrane, M_r 3500 cutoff) against aqueous acetic acid, and the solution concentrated in vacuo and then applied to a Sephadex G-50 SF column equilibrated with 10% aqueous acetic acid. Two almost completely separated peaks of peptide material were eluted from the column [Figure 1A of supplementary material (see paragraph at end of paper regarding supplementary material)]. On the basis of amino acid analyses and SDS–polyacrylamide gel electrophoresis, the first peak was identified as unreacted fragment FII and the second as fragment 228–316. Rechromatography of the combined fractions of the second peak on the same Sephadex column gave a symmetrical peak (Figure 1B of supplementary material), which was shown to contain a single component in SDS–polyacrylamide gel electrophoresis (Figure 2 of supplementary material). This technique indicated an approximate M_r of 10000, as expected for fragment 228–316. The experimental amino acid composition (Table I of supplementary material) compares well with that calculated from the known amino acid sequence (Titani et al., 1972). The NH_2-terminal residue of fragment 228–316 was determined by the Edman degradation method, and as expected, the phenylthiohydantoin of glycine was identified in the first cycle of degradation (experimental details not here reported). The yield of purified fragment 228–316 after lyophilization was 45%.

Circular Dichroism. The far-ultraviolet CD spectrum of fragment 228–316 at pH 7.8 (Figure 1A) shows two minima of negative ellipticity near 222 and 208 nm with a cross over

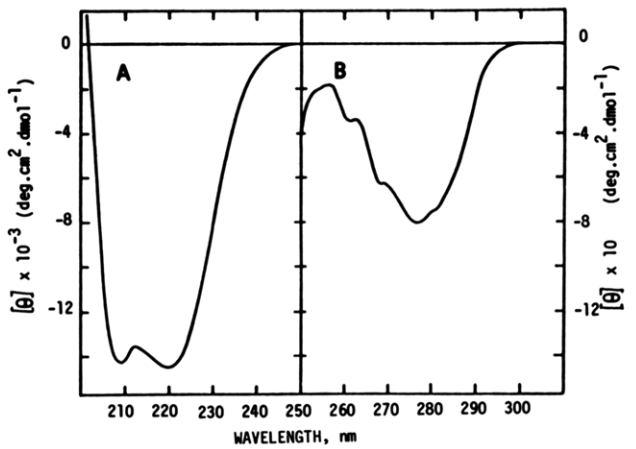

FIGURE 1: Far- (A) and near- (B) ultraviolet circular dichroism spectrum of fragment 228–316. Spectra were recorded at 22 °C in 10 mM Tris-HCl buffer, pH 7.8, containing 0.1 M NaCl at a fragment concentration of 0.19 and 1.06 mg/mL in the far- and near-ultraviolet region, respectively.

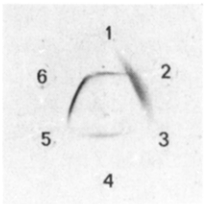

FIGURE 2: Double-immunodiffusion plate showing precipitin reactions of fragment 228–316, fragment 206–316, and native thermolysin against rabbit anti-thermolysin serum, on 1% agar in 20 mM Tris-HCl buffer, pH 7.4, containing 0.1 M NaCl and 10 mM $CaCl_2$. The center well contained 8 μL of anti-thermolysin serum, and the outer wells contained 8 μL of the following solutions in Tris buffer: 1, fragment 228–316 (0.6 mg/mL); 2, native thermolysin (0.3 mg/mL); 3, 2% bovine serum albumin; 4, fragment 228–316 (0.2 mg/mL); 5, 20 mM Tris-HCl buffer, pH 7.4, containing 0.1 M NaCl and 10 mM $CaCl_2$; 6, fragment 206–316 (0.4 mg/mL).

at 203 nm, indicating that a substantial portion of the polypeptide backbone is in an α-helical conformation (Greenfield & Fasman, 1969). In fact, quantitative analysis of the CD spectrum by the method of Siegel et al. (1980) indicates a content of 57% α-helical secondary structure. In the region of the side-chain absorption, the CD spectrum at 250–300 nm (Figure 1B) shows a negative trough centered at about 278 nm, which is related to the asymmetric environment of tyrosine and phenylalanine residues (Strickland, 1974) (the fragment does not contain tryptophan or cystine).

Immunochemistry. Anti-thermolysin antibodies have been elicited in rabbits with native thermolysin as antigen, and these have been used previously as conformational probes for similarity of structure between native thermolysin and fragment FII (Vita et al., 1982). Fragment 228–316 was tested in double-immunodiffusion experiments (Ouchterlony, 1948) together with thermolysin and fragment FII (Figure 2). Both fragments gave precipitin lines of nonidentity with native thermolysin and of qualitative identity to each other. The antigen–antibody reaction seems to be very specific and linked to conformational determinants, since denatured thermolysin (thermally denatured N_ϵ-succinylated thermolysin; Vita et al., 1979) fails to give precipitin lines in this double-immunodiffusion experiment. In quantitative immunoprecipitation studies, fragment 228–316 and fragment FII gave identical precipitin curves with anti-thermolysin serum (Figure 3). Both fragments gave a maximum of about 17% precipitation when compared with native thermolysin. These results es-

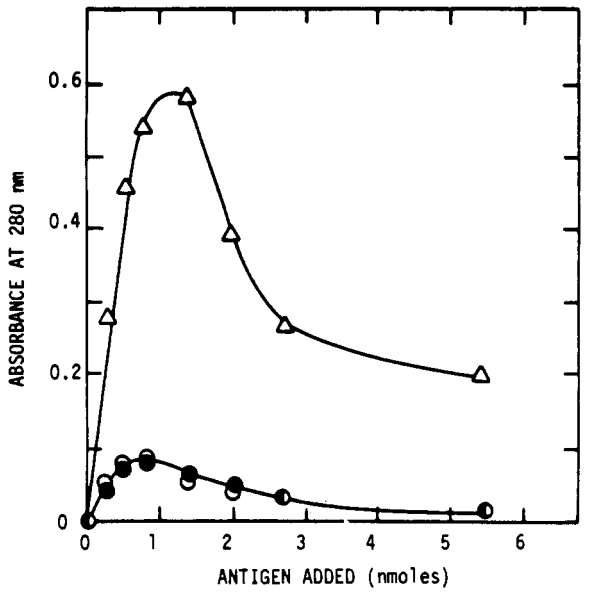

FIGURE 3: Quantitative precipitin reactions of thermolysin (△), fragment 206–316 (○), and fragment 228–316 (●) with rabbit anti-thermolysin serum (see Experimental Procedures).

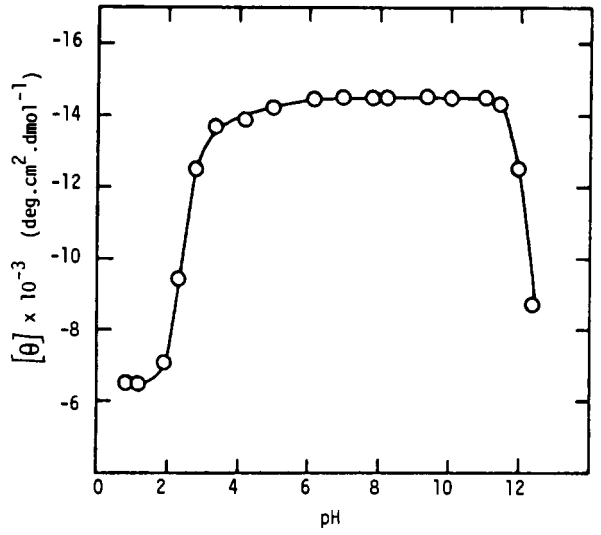

FIGURE 4: Effect of pH on the mean residue ellipticity, [θ], measured at 220 nm of fragment 228–316. The fragment was dissolved in 0.1 M NaCl at a concentration of 0.12 mg/mL, and the pH of the solution was adjusted to the desired pH with 1 N HCl or 1 N NaOH. When needed, dilution factors were considered. Measurements were carried out at 24 °C.

tablish with a high degree of confidence that both protein fragments interact identically with rabbit anti-thermolysin antibodies.

Stability. Far-ultraviolet CD spectra of fragment 228–316 were recorded at various pH values between pH 1 and 12. Examples of these spectra are given in Figure 3 of the supplementary material. These CD spectra show that conformational transitions take place in the fragment 228–316 at acid and alkaline pH. The plot of $[\theta]_{220nm}$ as a function of pH (Figure 4) shows that there is little structural change in the pH range from 3 to 11.5. It can be seen that the $[\theta]_{220nm}$ parameter falls very steeply between pH 1 and 4, indicating a sharp cooperative unfolding of its helical secondary structure with an apparent pK of 2.5.

Figure 5A shows the effect of the temperature on $[\theta]_{220nm}$ of fragment 228–316 dissolved in 10 mM Tris-HCl buffer, pH 7.8 containing 0.1 M NaCl. It is seen that on going from 25 to 85 °C the fragment displays a cooperative transition in the

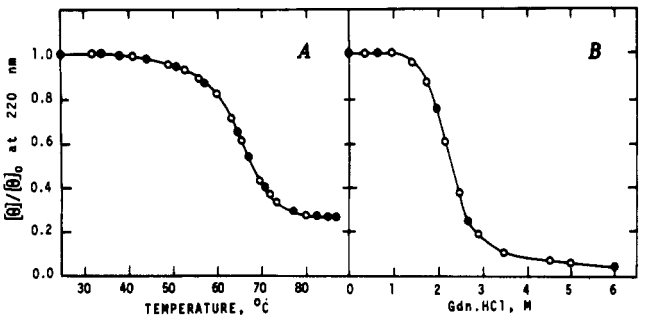

FIGURE 5: Heat-mediated and guanidine hydrochloride mediated unfolding of fragment 228–316 as monitored by far-ultraviolet circular dichroism measurements. (A) Effect of temperature on the mean residue ellipticity, [θ], measured at 220 nm of fragment 228–316 (0.19 mg/mL) dissolved in 10 mM Tris-HCl–0.1 M NaCl buffer, pH 7.8. The results are reported as $[\theta]/[\theta]_0$ as a function of temperature, where $[\theta]_0$ is the mean residue ellipticity at 220 nm measured at 20 °C. The results obtained by increasing (○) or decreasing (●) the temperature are shown. (B) Effect of increasing concentrations of guanidine hydrochloride (Gdn·HCl) on the mean residue ellipticity, [θ], measured at 220 nm of fragment 228–316 (0.07 mg/mL) dissolved in 20 mM sodium phosphate buffer–0.1 M NaCl, pH 8.3. Measurements were carried out at 24 °C after 10-min equilibration of the solutions. The results are reported as $[\theta]/[\theta]_0$ as a function of Gdn·HCl concentration, where $[\theta]_0$ is the mean residue ellipticity at 220 nm measured in phosphate buffer only. (○) Forward solutions; (●) backward solutions.

temperature range 50–70 °C, with a T_m (temperature of half-denaturation) of 66 °C. Upon being cooled, the thermal transition was shown to be fully reversible.

Exposure of fragment 228–316 to increasing concentrations of Gdn·HCl results in a cooperative reversible transition between 1 and 3 M, as monitored by $[\theta]_{220nm}$ (Figure 5B). Reversibility of the unfolding process is shown by the fact that the ellipticity values determined either by increasing or decreasing the denaturant concentration fall in the same curve. A midpoint denaturation is seen at 2.2 M Gdn·HCl. The equilibrium apparently was rapidly attained, since no time-dependent effects were seen during these CD measurements.

Considering that CD spectra in the far- and near-ultraviolet region are related to the secondary (Greenfield & Fasman, 1969) and tertiary (Strickland, 1974) structure of a polypeptide chain, respectively, the thermal unfolding of fragment 228–316 was followed also by monitoring the temperature dependence of the CD signal at 278 nm. Minor temperature effects on $[\theta]_{278nm}$ are seen below 50 °C, whereas a dramatic change in the ellipticity value occurs above this temperature (at 60–70 °C). A complete profile of $[\theta]_{278nm}$ vs. temperature was not obtained (Figure 4 of supplementary material), since at high temperature (~70 °C) a slight precipitation of the fragment occurred. It should be noted also that precipitation can be taken as a physical criterion of denaturation of a polypeptide chain. Thus, even if a precise figure of the temperature of half-denaturation could not be obtained from near-ultraviolet CD data, it is clear that the thermal unfolding of the secondary (Figure 4A) and tertiary (Figure 4 of supplementary material) structures of the fragment occurs in the same temperature range (60–70 °C).

Discussion

The results of this study provide evidence that nativelike structure is regenerated in the isolated fragment 228–316 of thermolysin. Both far-ultraviolet CD data and immunochemical cross-reactivity with antibodies elicited by native thermolysin are taken as supportive evidence. An estimate of 57% helical structure attained by the fragment in aqueous solution at neutral pH was obtained by a quantitative analysis of the far-ultraviolet CD spectrum of the fragment. This CD

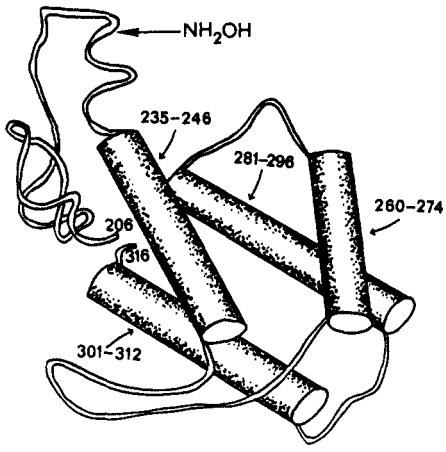

FIGURE 6: Schematic view of the tertiary structure of fragment 206–316 in intact thermolysin [adapted from Colman et al. (1972)]. The arrow indicates the Asn^{227}–Gly^{228} peptide bond cleaved by hydroxylamine. Numbers indicate the helical segments. β-Structure is absent.

analysis was performed by the computer-assisted method of Siegel et al. (1980), which provides an accurate calculation of the fraction of helical structure. The theoretical (Chen et al., 1974; Chang et al., 1978; Baker & Isemberg, 1976) and experimental (Konno et al., 1975; Wu & Yang, 1976; Hennessey & Johnson, 1981) limitations of methods proposed for quantitation of CD spectra are well-known and do not need to be recalled here. Nevertheless, we are inclined to conclude that the CD data are in good agreement with the expected amount of 62% helical structure for a native-like structure of the fragment, calculated on the basis of the helix content observed by X-ray diffraction methods in the corresponding region 228–316 in native thermolysin (Colman et al., 1972). CD data indicate that fragment 228–316 attains in solution a higher percentage of helicity with respect to fragment FII. This last fragment shows a 47% helicity calculated (Siegel et al., 1980) from the far-ultraviolet CD spectrum, which compares favorably with the 49.5% figure calculated from the X-ray structure of the parent protein (Colman et al., 1972). These results are consistent with the fact that segment 206–227 does not possess regular secondary structure in the native protein (Colman et al., 1972) (Figure 6; see also Figure 5 of supplementary material), and thus, its removal from fragment FII would of course enhance the percentage of helix, if a nativelike structure is maintained in the shortened fragment.

The most conclusive evidence for an independent folding of fragment 228–316 to a nativelike structure was obtained by immunochemical techniques. This is a well-recognized approach in biochemical literature, owing to the high degree of specificity of the antigen–antibody reaction (Atassi & Saplin, 1968; Sachs et al., 1972; Crumpton, 1974; Lazdunski, 1976; Habeeb, 1977). We previously prepared precipitating antibodies to native thermolysin and showed that these antibodies failed to precipitate heat-denatured N_ϵ-succinylated thermolysin (Vita et al., 1979), indicating that these antibodies were largely specific for conformational antigenic determinants. The recognition and precipitation of anti-thermolysin antibodies observed with fragment 228–316 support a close structural relationship between the fragment and intact native thermolysin. Double-immunodiffusion and quantitative precipitation experiments allowed the conclusion that fragment 228–316 interacts with the antibodies similarly to fragment FII (Figures 2 and 3), which was previously shown by quantitative binding experiments (competitive radioimmunoassays) to be indeed folded into a native structure with similar antigenic determinants as native thermolysin (Vita et al., 1982). Even if the immunochemical techniques used in this study did not permit a quantitative estimate of the relatedness of the structure of fragment 228–316 with that of the corresponding region in native thermolysin, it is clear that this fragment shares major antigenic dererminants with fragment FII and that these are not located in segment 206–227.

From the effect of pH on the ellipticity at 220 nm (Figure 4), it appears that the conformation of the fragment is stable over a broad pH range, whereas a sharp conformational transition occurs in the acid pH range, with a midpoint transition near pH 2.5. Unfolding of the fragment occurs also in the alkaline pH range, but only above pH 11.5 (Figure 4). A relatively high number of salt bridges has been observed in the structure of thermolysin, located in both lobes of the protein (Colman et al., 1972), and it has been proposed that these electrostatic interactions represent an important factor favoring the unusual thermostability of this thermophilic protein (Colman et al., 1972; Matthews et al., 1974; Holmes & Matthews, 1982). It is therefore expected that a similar network of salt bridges should contribute to the conformation and stability of fragment 228–316 in aqueous solution under neutral conditions, if a nativelike structure is attained in this isolated fragment. Four salt bridges occur in fragment 228–316 in the nativelike conformation, these involving aspartic and glutamic acid residues and lysine and arginine residues (Colman et al., 1972). Without ignoring the major role of hydrophobic interactions in protein folding and stability (Kauzmann, 1959), one may expect that these salt bridges stabilize the helical structure of the fragment quite strongly and that their disruption occurs only at rather extreme pH values, as indeed it is found (Figure 4). Thus, the acid transition undoubtedly results from the titration of carboxyl groups with anomalous low pK, as would be expected for groups involved in salt bridges (Scheraga, 1963).

In an earlier study (Vita & Fontana, 1982), we have shown that fragment FII unfolds by the action of heat, urea, and Gdn·HCl with a high degree of cooperativity. The thermodynamic parameters characterizing the unfolding of fragment FII, calculated with the assumption of a two-state transition, were shown to be typical of a small globular protein. In the case of fragment 228–316 we again find rather sharp, i.e., cooperative, unfolding transitions characterized by full reversibility. The thermal stability of fragment 228–316 is quite high (T_m = 66 °C) and identical with that previously observed with fragment FII. Also, the Gdn·HCl-mediated unfolding is similar for the two fragments, since the half-denaturation of fragment 228–316 occurs at 2.2 M denaturant concentration and that of fragment FII at 2.0 M (Vita & Fontana, 1982). These results indicate that a similar stable core is present in both fragments and that folding of the 228–316 portion of fragment FII appears to be thermodynamically independent of the 22 residues at the amino terminus.

Figure 6 shows a schematic drawing of fragment FII in a nativelike conformation (Matthews et al., 1972, 1974; Colman et al., 1972). The main feature of this structure is given by a bundle of four closely packed α-helices and no β-structure. This is a quite recurring protein structural motif observed in several sequentially and functionally unrelated proteins of relatively low molecular weight, such as myohaemerythrin (Ward et al., 1975), tobacco mosaic virus coat protein (Bloomer et al., 1978), apoferritin monomer (Banyard et al., 1978), *Escherichia coli* cytochrome *b*-562 (Mathews et al., 1980), cytochrome *c′* from *Rhodospirillum molischianum* (Weber et al., 1980), and uteroglobin (Mornon et al., 1980).

In addition, there has been considerable interest in the packing and strereochemistry (Weber & Salemme, 1980) of these tetrahelical complexes and in their folding pathways (Ptitsyn & Rashin, 1973; Richmond & Richards, 1978; Ptitsyn & Finkelstein, 1980; Lim, 1980). Thus, since CD and immunochemical measurements (see above) indicate that fragment 228–316 maintains a stable nativelike structure in aqueous solution at neutral pH, it can be proposed that also this fragment is organized as a four-α-helical bundle. On this basis, studies on the folding properties of fragment 228–316 may be useful for a better knowledge of this peculiar structure, which appears to be characterized by significant stability. In fact, the stability to heat and Gdn·HCl of fragment 228–316 (see Figure 5) is quite high, considering that, at variance from the above-listed tetrahelical proteins, this fragment is a single polypeptide chain of 89 amino acid residues lacking disulfide cross-links, cofactors, and strongly bound metal ions, all factors well-known to make significant contributions to the folding and stability of polypeptide chains.

Taking together the results of this study and those of previous ones (Vita et al., 1979, 1982; Vita & Fontana, 1982; Fontana et al., 1983), it appears that the NH_2-terminal portion 206–227 in fragment FII plays a minor role or no role at all in the nativelike folding and stability of that region (206–316). This is in line with the assumption of a nativelike structure of fragment FII (Figure 6), since segment 206–227 appears to lie outside the bundle of the four closely packed helices, thus constituting an exposed and flexible loop. In addition, it may be recalled that the calculated limits of protein domains and subdomains generally coincide with limits of secondary structure (Crippen, 1978; Wodak & Janin, 1981; Janin & Wodak, 1983; see also Appendix). Indeed, preliminary experiments of limited proteolysis of fragment FII employing different proteases have indicated that the NH_2-terminal portion of this fragment is easily cleaved; these results will be reported separately (D. Dalzoppo, C. Vita, and A. Fontana, unpublished results).

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Supplementary Material Available

A figure showing elution profiles from a Sephadex G-50 SF column of fragment 228–316, a figure showing a slab gel SDS electrophoresis, a table of amino acid composition of the fragment, a figure showing far-ultraviolet CD spectra of the fragment at different pH, and a figure showing the thermolysin crystallographic structure (4 pages). Ordering information is given on any current masthead page.

Registry No. Thermolysin, 9073-78-3.

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Appendix: Prediction of Stabilities of Thermolysin Fragments

It has been suggested that stable fragments with nativelike conformation within proteins can be located on the basis of calculations of buried surface area (Rashin, 1979, 1981). Here, it will be shown that these calculations can be a useful tool in planning experimental studies of folding properties of protein fragments.

An estimate of the free energy ΔG_D^N corresponding to the stability of a protein fragment of molecular weight M_r without disulfide bonds can be obtained by using eq 1 (Rashin, 1984).

$$\Delta G_D^N = T\Delta S_{conf} - \Delta G_B \quad (1)$$

Here, ΔG_B is proportional to the buried surface area, B (Lee & Richards, 1971), assumed to be the same as that in the X-ray structure of the entire protein, and is given by

$$\Delta G_B = 0.022 \text{ kcal/(mol·Å}^2) \times B \quad (2)$$

The conformational entropy is estimated with

$$T\Delta S_{conf} = \begin{cases} 0.01612M_r & M_r < 2320 \\ -16.94 + 0.02341M_r & 2320 < M_r < 10370 \\ -60.64 + 0.02762M_r & M_r > 10370 \end{cases} \quad (3)$$

Effects due to stabilization by disulfides (Rashin, 1984) are not considered, since thermolysin has no disulfides (Titani et al., 1972).

Estimated standard error σ in $T\Delta S_{conf}$ is ~1 kcal/mol for $M_r < 2600$ and increases up to 3–5 kcal/mol for higher molecular weights. If the calculated $\Delta G_D^N = -a\sigma$, then the assumption of a Gaussian distribution of the error in $T\Delta S_{conf}$ gives for the probability that $\Delta G_D^N > 0$ (i.e., that the corresponding fragment is actually unstable in its nativelike conformation):

$$P = 1 - 1/(2\pi)^{1/2} \int_{-a}^{\infty} \exp(-x^2/2)\, dx \quad (4)$$

On this basis, the stability of all subfragments of the cyanogen bromide fragments 1–120, 121–205, and 206–316 of thermolysin, previously investigated (Vita et al., 1979), was calculated according to eq 1–3.

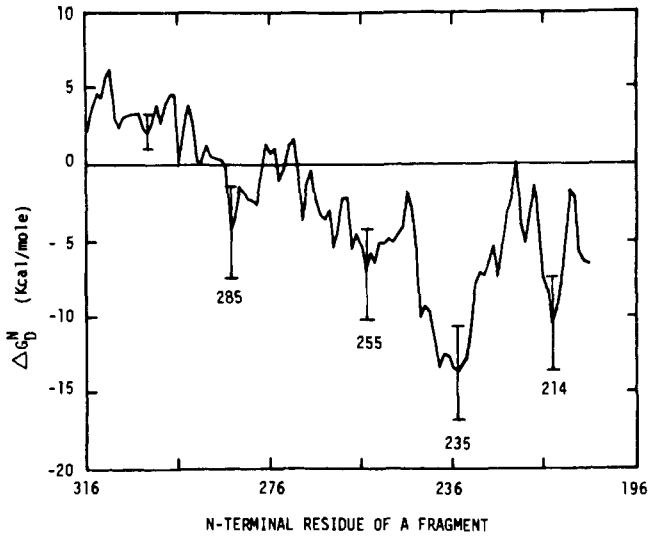

FIGURE A1: Calculated stabilities of subfragments of fragment 206–316 including the COOH-terminus of the thermolysin molecule. Numbers indicate NH_2-terminal residues of subfragments corresponding to stability minima. Vertical bars indicate standard errors.

No subfragments (except perhaps fragment 77–100) with stability better than the marginal value of –2 kcal/mol were found for fragments 1–120 and 121–205. The calculated stability of fragment 77–100 equals –3 kcal/mol. On the other hand, minima corresponding to calculated stabilities between –4 and –13.5 kcal/mol were found for fragment 206–316. This is in agreement with previously reported (Vita et al., 1979) far-ultraviolet circular dichroism studies on these fragments, which indicated a highly folded structure in fragment 206–316 and only residual structure in both fragments 1–120 and 121–205 in the absence of calcium ions (ion stabilization is not included in the calculations).

The calculated stabilities for all subfragments of fragment 206–316 starting at the COOH-terminus of the molecule are shown in Figure A1 (all fragments corresponding to stability minima include the COOH-terminus). The difference in stability between fragments 214–316 and 235–316 is within the accuracy of the calculations. If a standard error of σ = 3 kcal/mol is assumed, then the probabilities (P) for conformational unstability of subfragments of fragment 206–316 estimated according to eq 4 are P(285–316) = 0.076, P(255–316) = 0.0089, P(235–316) = 0.0000, and P(214–316) = 0.0002. Even with a standard error of σ = 5 kcal/mol, the corresponding probabilities are, in the same order, 0.19, 0.078, 0.0031, and 0.022. Thus, these calculations predict that fragments 255–316, 235–316, and 214–316 have a very good chance to fold into a nativelike and stable conformation. The probability that fragment 285–316 possesses a nativelike and stable conformation is lower than for the three longer fragments, but is still relatively high.

These predictions have prompted the experimental search for subfragments of fragment 206–316 possessing stable nativelike conformation. Experimental evidence for stability, cooperative unfolding, and nativelike structure in fragment 228–316 (containing the predicted fragment 235–316) is given under Experimental Procedures). Analogous results have been obtained for fragment 255–316, isolated after limited proteolysis of fragment 206–316 with subtilisin (D. Dalzoppo, C. Vita, and A. Fontana, unpublished results).

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CO Bond Angle Changes in Photolysis of Carboxymyoglobin†

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ABSTRACT: Previous studies [Chance, B., Fischetti, B., & Powers, L. (1983) *Biochemistry 22*, 3820–3829] of the local structure changes around the iron in carboxymyoglobin on photolysis at 4 K revealed that the iron–carbon distance increased ~0.05 Å but was accompanied by a lengthening of the iron–pyrrole nitrogen bonds of the heme (~0.03 Å) that was not as large as that found in the deoxy form. Further analysis of these data together with comparison to model compounds indicates that the Fe–C–O bond angle in carboxymyoglobin is bent (127 ± 4°), having a structure identical, within the error, with the "pocket" porphyrin model compound FePocPiv(1-MeIm)(CO) [Collman, J. P., Brauman, J. I., Collins, T. J., Iverson, B. L., Lang, G., Pettman, R., Sessler, J. L., & Walters, M. A. (1983) *J. Am. Chem Soc. 105*, 3038–3052]. On photolysis, this angle decreases by 5–10°. In addition, correlation is observed between the increase in the length of the Fe–C bond and the decrease of the Fe–C–O angle. These results suggest that the rate-limiting step in recombination is the thermal motion of CO in the pocket to achieve an appropriate bonding angle with respect to the iron. These changes constitute the first molecular picture of the photolysis process, as well as the structure of the geminate state, and are important in clarifying nuclear tunneling parameters.

The photolysis and recombination of carboxymyoglobin at 4 K have been shown to involve a displacement of the CO by ~0.05 Å accompanied by expansion of the iron–pyrrole nitrogen bonds of the heme (Fe–N_p) of ~0.03 Å and possibly lengthening of the iron–proximal histidine (Fe–N_ϵ) bond by ~0.02 Å (Chance et al., 1983). This Fe–N_p average distance is less than that observed for deoxymyoglobin (Mb) with the Fe–N_ϵ distance ~0.10 Å longer (Chance et al., 1983; Takano, 1977). Thus, the structure of this geminate-state photoproduct (Mb*CO) differs significantly from that of Mb. Whatever the molecular origin of this difference, the motion of the CO in recombination to form MbCO must be a step of low activation energy (Austin et al., 1975), producing a small change in the charge density of the iron and a small change in the Fe–C distance (Chance et al., 1983). Rotation of the CO from a preferred position was suggested by Hush (Grady, et al., 1978) from octapole moment studies of CO compounds and by Alben et al. (1983) from studies of the CO stretching frequencies. The possibility that an Fe–C–O bond angle change occurs on photolysis, which may provide the rate-limiting step in recombination, has not been considered.

Although crystallographic studies of small molecules and models having terminal CO groups show the Fe–C–O bond to be nearly linear (Peng & Ibers, 1976; Hoard, 1975), Fe–C–O angles of 135–145° have been observed in several carboxy hemoproteins, including erythrocruorin (Huber et al., 1970), bloodworm *Glycera dibranchiata* (Padian & Love, 1974), horse hemoglobin (Heidner et al., 1976), and myoglobin (Norvell et al., 1975). Presented here are EXAFS (extended X-ray absorbance fine structure) studies of a variety of model compounds and corresponding proteins at 4 K, which are compared to those of carboxymyoglobin and its photoproduct (Chance et al., 1983). We conclude that carboxymyoglobin has an Fe–C–O bond angle of 127 ± 4° and an identical structure within our error with that of the "pocket" porphyrin model FePocPiv(1-MeIm)(CO) (Collman et al., 1983a). This angle decreases 5–10° on photolysis at 4 K.

Materials and Methods

Sample Preparation, Cryogenic Technique, and Sample Monitoring. $Ni(CO)_4$, $Na_2Fe(CO)_4$, and $Fe(CO)_5$ were purchased commercially at the highest purity available and used without further purification. Fe(TPP)(Py)(CO) was prepared by the method of Peng & Ibers (1976), the "pocket" porphyrin FePocPiv(1-MeIm)(CO) by the method of Collman et al. (1983a), and the "picket fence" porphyrin FeTpivP(1-MeIm)(CO) by the method of Collman et al. (1975). MbO_2, met-Mb, and HbO_2 were prepared by methods similar to those reported for MbCO (Chance et al., 1983). Cryogenic techniques and sample monitoring by optical spectroscopy were identical with those reported in detail for MbCO (Chance et al., 1983).

Data Analysis. Data were analyzed by procedures discussed previously (Powers et al., 1981; Lee et al., 1981; Chance et al., 1983) and included background subtraction of the "isolated atom" contribution and k^3 (wave vector) multiplication followed by Fourier transformation. The respective coordination shell contributions were isolated by Fourier filter and back-transformation.

The filtered data of the first shell contributions were then fit by a two atom type procedure (Lee et al., 1981; Peisach

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