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Hierarchic organization of globular proteins: Experimental studies on thermolysin

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Abstract. Previous studies from this laboratory have shown that the thermolysin fragment 121-316, comprising entirely the "all-α" COOH-terminal structural domain 158–316, as well as fragment 206–316 (fragment FII) are able to refold into a native-like, stable structure independently from the rest of the protein molecule. The present report describes conformational properties of fragments 228–316 and 255–316 obtained by chemical and enzymatic cleavage of fragment FII, respectively. These subfragments are able to acquire a stable conformation of native-like characteristics, as judged by quantitative analysis of secondary structure from far-ultra-violet circular dichroism spectra and immunochemical properties using rabbit anti-thermolysin antibodies. Melting curves of the secondary structure of the fragments show cooperativity with a temperature of half-denaturation (T_m) of 65–66°C. The results of this study provide evidence that it is possible to isolate stable supersecondary structures (folding units) of globular proteins and correlate well with predictions of subdomains of the COOH-terminal structural domain 158–316 of thermolysin.

Keywords. Circular dichroism; immunochemistry; protein domains; protein fragments; thermolysin.

Introduction

The word domain of a protein is currently used to indicate large subassemblies of secondary structure elements which appear tightly packed in the crystal structure of globular proteins. Visually recognized blocks or folded units are commonly referred to as structural domains to indicate that they are identified by inspection of crystallographically determined protein structures. Wetlaufer (1973) was the first investigator to emphasize the structural role of domains in globular proteins and to propose that domains could represent intermediates in the folding process of protein molecules. As schematically shown in figure 1, it is conceivable to suggest that specific segments of an unfolded 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 (Jaenicke, 1984). The major implication of this model of protein folding by a mechanism of modular assembly is that isolated protein fragments corresponding to domains in the intact protein are expected to fold into a native-like structure independently from the rest of the polypeptide chain, thus resembling in their properties a small mol wt globular protein (Vita and Fontana, 1982). Indeed, this possibility is being tested experimentally in a number of laboratories using fragments of

Abbreviation used: CD, Circular dichroism.

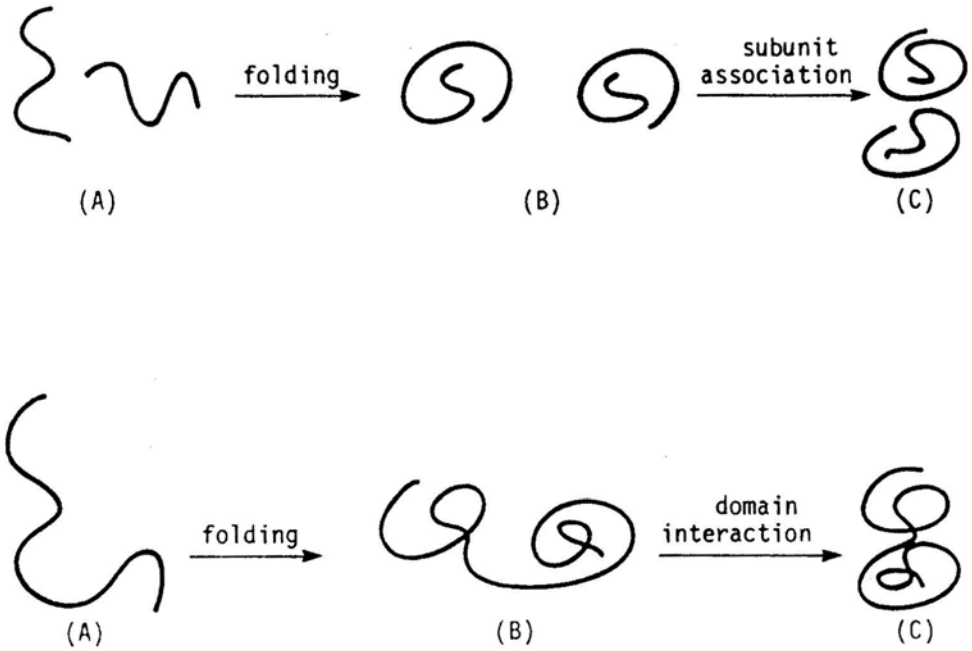

Figure 1. Schematic representation of stages of folding in an oligomeric protein (top) or multi-domain protein (bottom). The example shown is for a protein with two subunits or for a single-chain protein with two domains. A, represents the unfolded state; B, illustrates formation of an intermediate folded state constituted by isolated subunits (top) or domains (bottom); C, folded, biologically active structure formed as a result of subunit association (top) or domain interaction (bottom).

large proteins obtained by chemical cleavage or limited proteolysis (cf. for references Wetlaufer, 1981; Rossmann and Argos, 1981; Janin and Wodak, 1983).

Since location of structural domains by visual inspection of protein models suffers from subjectivity, quantitative methods involving computer algorithms were proposed to identify domains from X-ray elucidated coordinates (Crippen, 1978; Rose, 1979; Wodak and Janin, 1981; Rashin, 1981). Using algorithm methods, it was shown that the visually recognized large domains (Wetlaufer, 1973) are decomposable into sub-domains, and that these entities are, in turn, further decomposable, and so forth (Crippen, 1978; Rose, 1979). Thus, as shown in figure 2, these algorithms allowed a description of globular proteins in terms of a hierarchic architecture, ranging from elements of secondary structure to the whole protein (Schulz, 1977). These analyses of the static crystal structure of proteins were used to propose folding pathways involving the hierarchic condensation of continuous chain modules of the polypeptide chain (folding tree) (Crippen, 1978; Rose, 1979; Rashin, 1979; Lesk and Rose, 1981).

A few years ago, we undertook the study of chemical fragments of thermolysin in order to address the question of the existence, location and properties of protein domains (Vita *et al.*, 1979, 1982, 1983, 1984; Vita and Fontana, 1982; Fontana *et al.*, 1983). Thermolysin shows a quite peculiar bilobal morphology, with two distinct

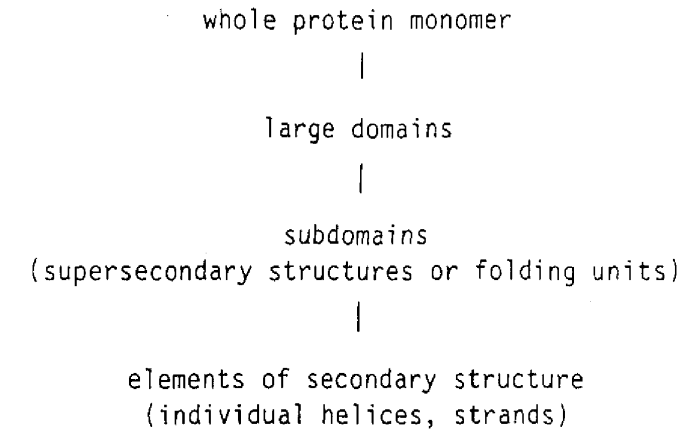

Figure 2. Hierarchic organization of globular proteins.

structural domains of equal sizes (residues 1–157 and 158–316) (Wetlaufer, 1973) and the active site located at the interface between them (Matthews *et al.*, 1972, 1974; Colman *et al.*, 1972; Holmes and Matthews, 1982). We have previously shown that the cyanogen bromide fragment 121–316 (Vita *et al.,* 1983), comprising entirely the "all-α" (Levitt and Chothia, 1976) COOH-terminal domain 158–316, as well as fragment 206–316 (fragment FII) (Fontana *et al.,* 1983) is able to refold into a structure of native-like characteristics, as judged by quantitative analysis of secondary structure from far-ultra-violet circular dichroism (CD) spectra and immunochemical properties using rabbit anti-thermolysin antibodies. We now report the isolation and characterization of fragments 228–316 and 255–316 obtained by chemical and proteolytic cleavage of fragment FII, respectively, with the aim to define the minimum size of its polypeptide chain still able to acquire a stable native-like conformation. Both fragments were found to be capable of independent folding in aqueous solution at neutral pH. These results demonstrate that it is possible to isolate supersecondary structures (subdomains or folding units) from globular proteins and corroborate the predicted location of subdomains in the COOH-terminal domain of thermolysin (Crippen, 1978; Wodak and Janin, 1981; Rashin, 1984; Rose, G. D., private communication).

Chemical and proteolytic cleavage of fragment FII (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 (Bornstein and Balian, 1977). After incubation of the fragment at 45°C for 5 h with 2 M NH_2OH in 6 M guanidine hydrochloride, pH 9·4, the reaction mixture was dialyzed and then applied to a Sephadex G-50SF column equilibrated and eluted with 10 % aqueous acetic acid. Two almost completely separated peaks of peptide material were eluted from the column, the first peak being unreacted fragment FII and the second fragment 228–316. Final purification of this last fragment was achieved by rechromatography on the same Sephadex column. The purity and identity of the fragment was established by

SDS-polyacrylamide gel electrophoresis and amino acid analysis after acid hydrolysis (Vita *et al.,* 1984).

Limited proteolysis of fragment FII by subtilisin was performed at a 200:1 (w/w) ratio of fragment to protease and the products of proteolysis analyzed by SDS-gel electrophoresis. As shown in figure 3, a major fragment of mol wt ~ 6000 is formed, preceded by a transient formation of fragments of intermediate mol wt. The main component of the proteolytic mixture obtained after 30 min digestion was purified by gel filtration on a Sephadex G-50SF column equilibrated with 10 % aqueous formic acid. The gel filtration chromatogram indicated that the ~ 6000 mol wt fragment was the major component, in agreement with the data obtained by SDS-polyacrylamide gel electrophoresis. Interestingly, besides the fragment, only rather small peptides and amino acids were eluted from the column, indicating that further proteolysis of the fragment leads to extensive degradation and not to fragments of intermediate size. The identity of the isolated thermolysin fragment was established by amino acid analysis after acid hydrolysis, determination of its NH_2 -terminal sequence after three steps of Edman degradation, as well as COOH-terminal sequence analyzing the time-course release of amino acids by carboxypeptidase Y digestion (Dalzoppo *et al.,* 1985). Comparison of all these data with the known amino acid sequence of thermolysin (Titani *et al.*, 1972) led us to conclude that the isolated fragment corresponds to sequence 255–316.

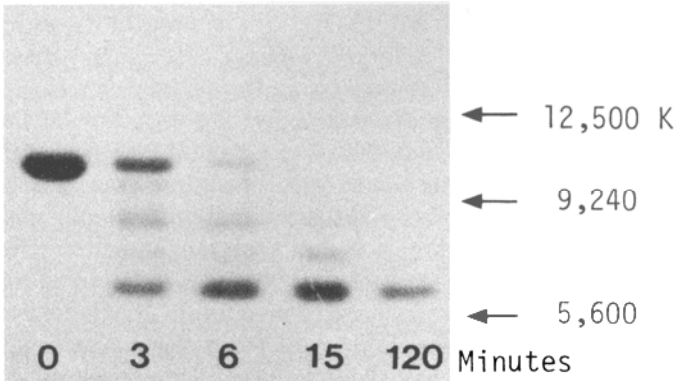

Figure 3. Limited proteolytic digestion of fragment FII of thermolysin as monitored by SDS-polyacrylamide gel electrophoresis of digestion products. Proteolysis was carried out at 22°C with subtilisin Carlsberg at 200:1 (w/w) ratio of fragment to protease in 20 mM Tris-HCl buffer, pH 9·0, containing 0·1 M NaCl. Aliquots (~ 10 μg of peptide material) were taken from the reaction mixture at intervals, immediately diluted with formic acid in order to stop proteolysis, dried *in vacuo* over NaOH pellets and then dissolved in the sample buffer for SDS-gel electrophoresis. Molecular weight markers are indicated in the right part of the figure.

Conformational and unfolding studies

Circular dichroism

In order to determine the secondary structure characteristics of fragment FII and its subfragments 228–316 and 255–316, the CD spectra in the far-ultraviolet region were

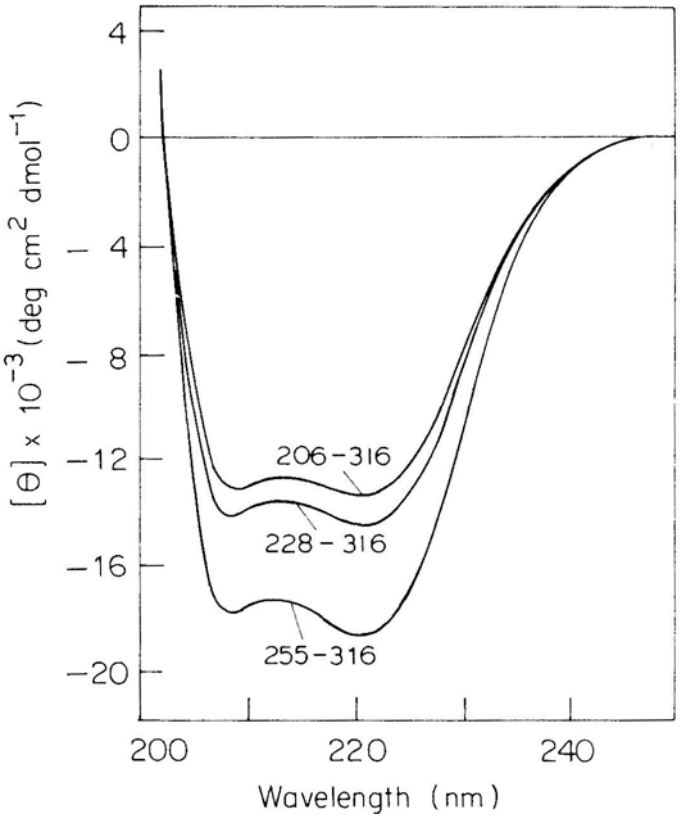

Figure 4. Far-ultraviolet circular dichroism spectra of thermolysin fragments FII, 228–316 and 255–316. Spectra were recorded at 22–23°C in 10 mM Tris-HCl buffer, pH 7·5, containing 0·1 M NaCl, at a fragment concentration of ~ 0·1 mg/ml.

examined in aqueous buffer at neutral pH. As shown in figure 4, the CD spectra of the fragments show two minima of negative ellipticity near 222 and 208 nm with a cross-over at 203 nm, indicating that a substantial portion of the polypeptide backbone is in an α-helical conformation (Greenfield and Fasman, 1969). Quantitative analyses of secondary structure from far-ultraviolet CD spectra were carried out using the method of Chen *et al.* (1972, 1974) and of Siegel *et al.* (1980). The figures thus obtained for the per cent *α*-helical content for the fragments are listed in table 1, together with the figures expected for a native-like structure of the fragments and calculated from the crystallographically determined structure of thermolysin (Matthews *et al.*, 1972, 1974; Colman *et al.*, 1972; Holmes and Matthews, 1982).

Immunochemistry

In order to assess the relationship between fragment conformation and the conformation of the corresponding regions in intact thermolysin, the fragments were studied with respect to their antigenic properties using antibodies elicited in rabbits using

Table 1. α-Helical content of carboxyl-terminal fragments of thermolysin as determined from far-ultraviolet CD spectra.

Fragment	Number of residues	α-Helix (%) X-Ray[a]	This study	
206–316	111	49·5	47[b]	45[c]
228–316	89	62·0	57[b]	53[c]
255–316	62	69·3	77[b]	63[c]

[a] Calculated on the basis of the helical content of the pertinent fragments in native thermolysin, as deduced from the X-ray structure of the protein (Matthews *et al.*, 1972, 1974; Colman *et al.*, 1972).
[b] Method of Siegel *et al.* (1980).
[c] Method of Chen *et al.* (1972, 1974)

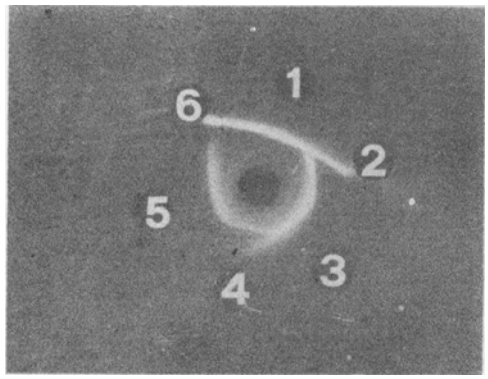

Figure 5. Double-immunodiffusion plate showing precipitin reactions of native thermolysin and fragments FII, 228–316 and 255–316 against rabbit anti-thermolysin serum on 1 % agar in 20 mM Tris-HCl buffer, pH 7·4, containing 0·1 M NaCl and 10 mM $CaCl_2$. The center well contained 8 μl of anti-thermolysin serum and the outer wells contained 8 μl of the following solutions in Tris buffer: 1, native thermolysin (0·6 mg/ml); 2, fragment FII (0·3 mg/ml); 3, fragments 228–316 (0·3 mg/ml); 4, fragment 255–316 (0·1 mg/ml); 5, fragment 255–316 (0·3 mg/ml); 6, 2 % bovine serum albumin.

native thermolysin as immunogen (Vita *et al.*, 1979). As indicated by immunodiffusion assays (figure 5), anti-thermolysin antibodies recognize and precipitate fragment FII and its subfragments. These antigen-antibody reactions were shown to be specific and linked to conformational determinants, since denatured thermolysin failed to give precipitin lines in this immunodiffusion experiment (Vita *et al.*, 1979).

Thermal unfolding

Fragment FII and its subfragments 228–316 and 255–316 were found relatively stable to the denaturing action of heat, being denatured only at relatively high temperature

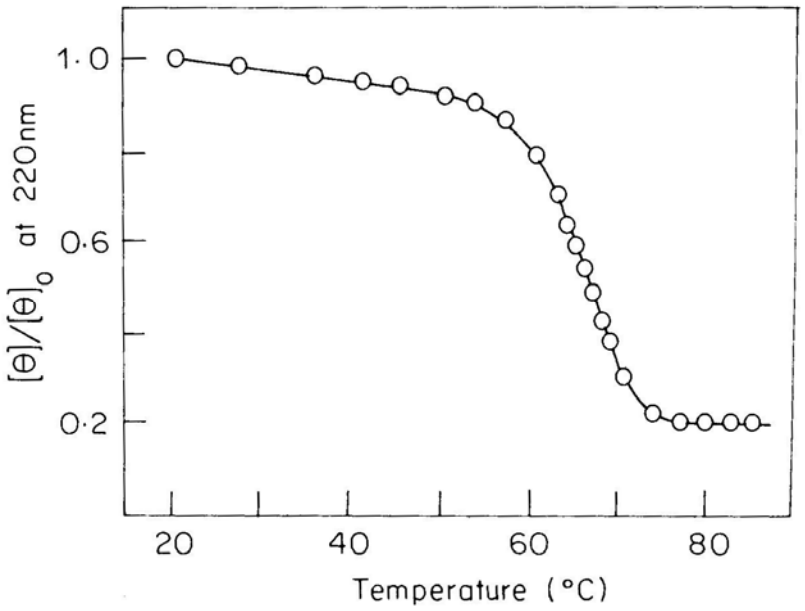

Figure 6. Thermal unfolding of fragment 255–316 of thermolysin monitored by circular dichroism measurements in the far-ultraviolet region. The fragment was dissolved (0·12 mg/ml) in 10 mM morpholinopropane-sulfonic acid, pH 7·8, containing 0·2 M NaCl. The results are reported as $[\theta]/[\theta]_0$ as a function of temperature, where $[\theta]_0$ refers to the mean residue ellipticity at 220 nm measured at 20°C.

(60–70°C). As an example, figure 6 shows the temperature dependence of the dichroic signal at 220 nm of fragment 255–316 in morpholinopropane sulphonate buffer, pH 7·8, containing 0·2 M NaCl. On going from 20 to 85°C a cooperative helix to coil transition with a T_m (temperature of half-denaturation) of 65°C is observed. Fragment FII and 228–316 displayed quite similar melting curves and a figure for T_m of 66°C was calculated in both cases (Vita and Fontana, 1982; Vita *et al.*, 1984).

Discussion

The results of this study provide evidence that native-like structure is regenerated in fragments 228–316 and 255–316 of thermolysin. Both far-ultraviolet CD data and immunochemical cross-reactivity with antibodies elicited by native thermolysin are taken as supportive evidences. Considering the theoretical (Baker and Isemberg, 1976; Chang *et al.*, 1978; Hennessey and Johnson, 1981) and experimental (Wu and Yang, 1976; Tuzimura *et al.*, 1977) limitations of methods of calculation of protein secondary structure from far-ultraviolet CD spectra, the figures of α-helix content of the fragments reported in table 1 indicate a moderate agreement between the experimentally determined helicity from far-ultraviolet CD spectra with that expected for a native-like structure of the fragments. Furthermore, the recognition and precipitation of anti-thermolysin antibodies observed with fragment FII and its subfragments

228–316 and 255–316 are indicative of a close structural relationship between fragments and intact native thermolysin. Thus, considering that antibodies elicited towards a globular protein are specific, far antigenic determinants which are located in the more exposed regions of the protein molecule, it can be concluded that important details (loops, corners) of three-dimensional structure are shared between the fragments and the native parent protein (for reviews on the use of antibodies as probes of conformation see Crumpton, 1974; Reichlin, 1975; Lazdunski, 1976).

The native-like structure of fragment 228 316 consists of a tetra-helical complex, a frequently recurring protein structural motif in sequentially and functionally unrelated proteins (Weber and Salemme, 1980) (figure 7). In the present case, the NH_2-terminal helix appears to be loosely connected to the other three helices, since it is quite easily cleaved off by subtilisin from the rest of the polypeptide chain. This is in line with the assumption of a native-like structure of fragment FII, since an analysis of the list of the salt bridges in native thermolysin (Colman *et al.*, 1972) and of the side chains of amino acid residues involved in their formation reveals that four salt bridges link together the three COOH-terminal helices contained in segment 255–316, whereas two salt bridges are found within segment 206–250.

Protein fragments possessing domain characteristics are expected to behave like small mol wt globular proteins, *i.e.* to show cooperativity of unfolding transitions. Indeed, fragment FII (Vita and Fontana, 1982) and its subfragments 228–316 and 255–316 show cooperativity of thermal unfolding (see figure 6). The thermal stability of the fragments (T_m 65–66°C) appears to be remarkable, if one considers their size and

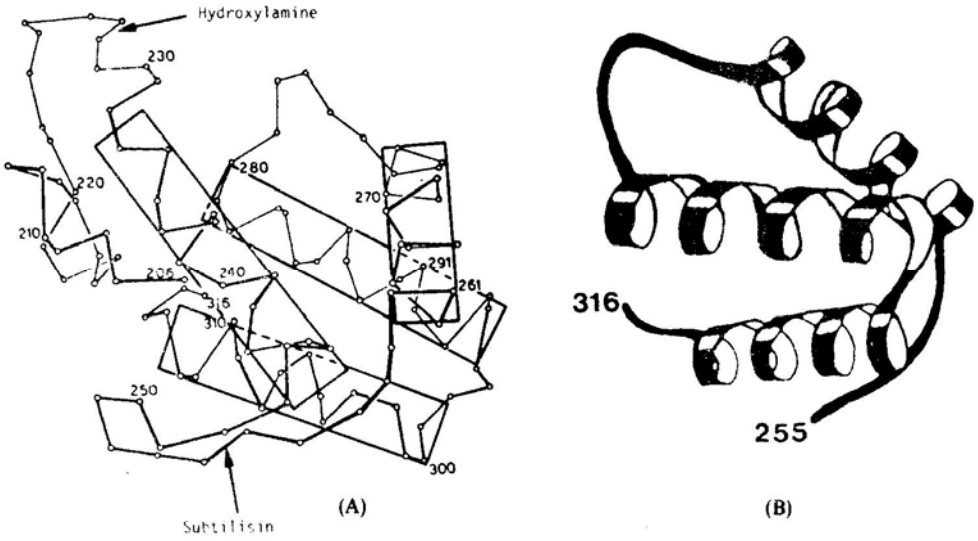

Figure 7. A. Polypeptide backbone conformation of fragment FII of thermolysin in the intact thermolysin (adapted from Matthews *et al.*, 1972). The four helices are indicated. Arrows indicate sites of cleavage by hydroxylamine and sultilisin, corresponding to the peptide fragments isolated and characterized (fragments 228–316 and 255–316). **B.** A simplified representation of the polypeptide backbone of fragment 255-316 in a native-like conformation. Helices are depicted as helical ribbons (adapted from Richardson, 1981).

the fact that they are simple polypeptide chains lacking disulphide bridges, cofactors, strongly bound ions, all characteristics well known to contribute significantly to the folding and stability of protein structures.

It is of interest to relate the results of this study to the predicted locations of subdomains in the COOH-terminal structural domain (residues 158–316) (Wetlaufer, 1973) of thermolysin. These predictions, based on the X-ray structure of thermolysin all identified a subdomain comprising roughly residues 240–316 (Crippen, 1978; Wodak and Janin, 1981; Rashin, 1984; Rose, G. D., private communication). Crippen (1978) computed a subdomain constituted by the assembly of three COOH-terminal helices (see figure 7), whereas helix 233–246 was found to interact with other parts of the polypeptide chain of intact thermolysin. Rashin (1984) computed the location of stable subdomains with native-like conformation in the COOH-terminal portion of the thermolysin molecule on the basis of surface area (Lee and Richards, 1971) measurements and predicted that subfragments 214–316, 235–316, 255–316 and perhaps also 285–316 should have a good chance to show independent folding. On the overall, the results of the present study are in essential agreement with these predictions.

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