Stability and Stabilization of Biocatalysts
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LIMITED PROTEOLYSIS OF PROTEINS BY THERMOLYSIN IN TRIFLUOROETHANOL

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We have examined the proteolysis of model proteins by thermolysin when dissolved in aqueous buffer at neutral pH in the presence of 50% (by vol.) trifluoroethanol (TFE). Under these solvent conditions, proteins acquire a new conformational state characterized by enhanced helical secondary structure, but lacking the specific tertiary interactions of the native species. It was found that the TFE-state of proteins dictates very selective peptide bond fissions by the TFE-resistant thermolysin, which otherwise shows broad substrate specificity. Nicked protein species with a single peptide bond hydrolyzed have been prepared and isolated to homogeneity in the case of bovine ribonuclease A (cleavage at Asn34-Leu35), hen lysozyme (Lys97-Ile98), bovine α-lactalbumin (Ala40-Ile41) and horse cytochrome c (Gly56-Ile57).

1. INTRODUCTION

Non aqueous enzymology has emerged as a very active and useful area of research in recent years, prompting fundamental questions concerning enzyme structure and dynamics and their effect on catalysis (Wong, 1989; Klibanov, 1989, 1997; Dordick, 1989; Mattiasson & Adlercreutz, 1991; Gupta, 1992; Carrea et al., 1995). The addition of water-soluble cosolvents may change dielectric constant, hydrophobicity and hydrogen bonding of the reaction medium, altering the various forces responsible for the substrate binding specificity (electrostatic and van der Waal's forces, hydrophobic and steric effects, hydrogen bonds) (Jencks, 1969; Fersht, 1985; Kraut, 1988) and thus the catalytic properties of the enzyme. In particular, the hydrogen bonding properties of alcohols, sugars and polyols are expected to change both the solvent medium and the solvation of protein molecules, thus leading to (subtle) conformational changes of an enzyme and its catalytic behaviour (Pourplanche et al., 1994; Ligné et al., 1997). In the case of proteolytic enzymes, the cosolvent can alter the conformational properties of

Abbreviations: CD, circular dichroism; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TFE, trifluoroethanol; HPLC, high performance liquid chromatography; [θ], mean residue ellipticity; nicked protein, a protein species with a peptide bond hydrolyzed.

both the enzyme and the peptide/protein substrate and consequently their mutual interaction. The use of proteolytic enzymes in the presence of organic solvents has been extensively investigated in the past and successfully utilized for the synthesis or semi-synthesis of peptides and proteins, since protease, in the presence of water-soluble cosolvents (glycerol, 2-propanol) catalyze the reverse reaction, *i.e.* the synthesis instead of hydrolysis of peptide bonds (Chaiken, 1981; Kullman, 1987; Wayne & Fruton, 1983; De Filippis & Fontana, 1990).

Short-chain alcohols such as methanol, ethanol, propanol, chloroethanol and, in particular, trifluoroethanol (TFE) have been shown to induce and stabilize α-helical structure in otherwise randomly coiled or partially structured polypeptides (Tamburro et al., 1968; Nelson & Kallenbach, 1986; Lehrman et al., 1990; Storrs et al., 1992; Sönnichsen et al., 1992). TFE is nowadays the cosolvent of choice for enhancing the helical secondary structure of polypeptides (Smith et al., 1994; Cammers-Goodwin et al., 1996; Bolin et al., 1996; Luo & Baldwin, 1997; Myers et al., 1998). The helix-inducing effect of TFE does not seem to occur independently of the amino acid sequence of the polypeptide chain, since peptides and protein fragments corresponding to helical regions in the native protein have a higher tendency to form a helix in the presence of TFE (Lehrman et al., 1990; Segawa et al., 1991). However, also protein fragments derived from predominantly β-sheet proteins acquire a helical structure in the presence of TFE (Fan et al., 1993; Hamada et al., 1995; Jayaraman et al., 1996).

TFE has been shown also to disrupt the native conformation of globular proteins (Shiraki et al., 1995, and references cited therein), but the resulting denaturated state is much different from the random-coiled state observed in the presence of chemical denaturants such as urea or guanidine hydrochloride. Recent studies have shown that TFE-state of a protein, *e.g.* as obtained by dissolving the protein (lysozyme, α-lactalbumin) in 50% (by vol.) aqueous TFE, is a stable, partially folded state with a high content of α-helical conformation, but lacking the specific tertiary interactions of the native protein (Buck et al., 1993, 1995, 1996; Alexandrescu et al., 1994). This TFE-state appears to be a non-compact, expanded conformational state characterized by an ensemble of fluctuating helices. However, the TFE-state does not result from a gross structural reorganization of the protein to another unrelated structure, since the large majority of amides protected from exchange in the TFE-state are also protected in the native state of the protein, thus implying a similar location of helical segments along the polypeptide chain in both native and TFE-state (Buck et al., 1993, 1995, 1996; Alexandrescu et al., 1994).

In recent years we have been interested at demonstrating that proteolytic enzymes can be used as reliable probes of protein structure and dynamics (Fontana et al., 1986, 1993, 1997a,b). The outcome of these studies allowed us to propose that the key feature of the sites of initial proteolysis of a globular protein substrate resides in the enhanced flexibility (local unfolding) and, in particular, that helical chain segments are not prone to proteolysis (Polverino de Laureto et al., 1995a; Fontana et al., 1997a,b). On this basis, it was expected that the highly helical conformation of a protein substrate dissolved in aqueous TFE would hampers extensive proteolysis. We have conducted

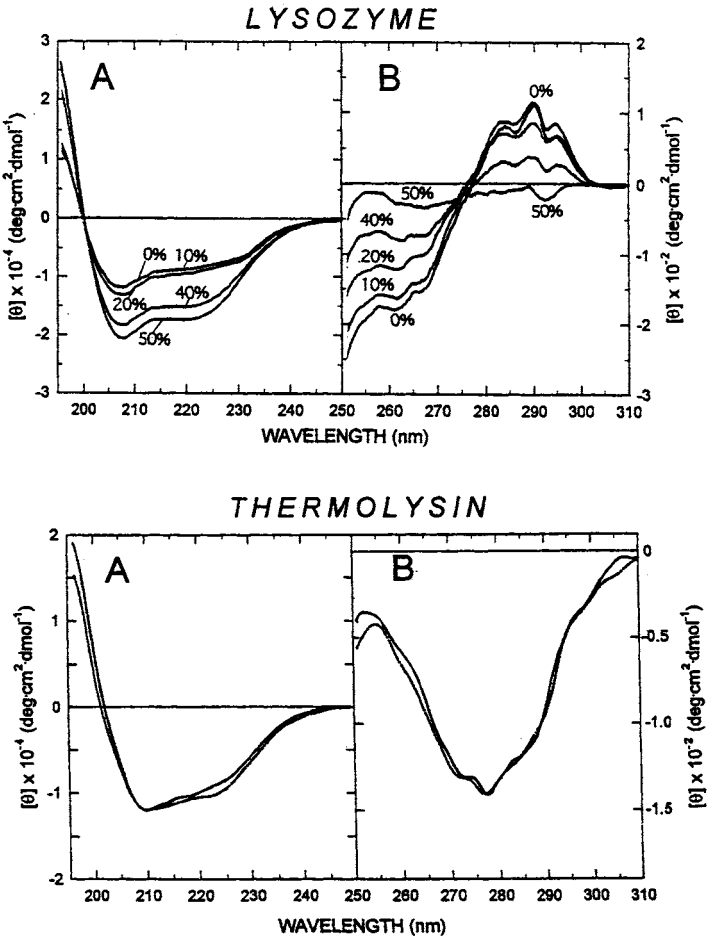

Figure 1. Far-UV (A) and near-UV (B) circular dichroism (CD) spectra of hen egg-white lysozyme (top) and thermolysin (bottom). The spectra of lysozyme were recorded at room temperature in 20 mM sodium phosphate buffer, pH 7.0, containing 50 mM NaCl and various amounts of TFE. The numbers near the curves indicate the % of TFE. In the case of thermolysin, the CD spectra were recorded in 20 mM Tris-HCl buffer, pH 7.2, containing 5 mM $CaCl_2$ in the absence (—) or presence of 50% TFE(--). Protein concentrations were about 0.1 and 0.8 mg/ml in the far- and near-UV regions, respectively.

proteolysis of several model proteins in their TFE-state utilizing thermolysin (Fontana et al., 1995; Polverino de Laureto et al., 1995b,c; 1997). This thermophilic metallo-endopeptidase (Matthews, 1988) appeared to be a most suitable proteolytic probe, because of its noteworthy stability under relatively harsh solvent conditions, including organic solvents (Welinder, 1988), and broad substrate specificity (Morihara & Tzusuki, 1970; Heinrikson, 1977; Keil, 1982). Thus, it was anticipated that thermolysin would cleave proteins in their TFE-state at sites characterized by the flexibility required for an

efficient proteolysis and not by the specificity of the protease. The striking observation emerged from these studies is that proteolysis by thermolysin in 50% TFE can be very selective, indicating that this novel procedure can be useful for preparing nicked proteins and/or large protein fragments.

2. PROTEINS IN 50% TFE

The conformational properties of four model proteins (hen egg-white lysozyme, bovine pancreatic ribonuclease A, bovine α-lactalbumin and horse cytochrome c) dissolved in aqueous TFE were examined by far-UV circular dichroism (CD) spectroscopy. As an example, Fig. 1 shows the CD spectra of lysozyme in aqueous buffer, pH 7.0, in the presence of increasing concentrations of TFE. The features of the far- and near-UV CD spectra clearly show that the action of alcohol is to enhance the helical secondary structure of the protein, as deduced by the shape of the CD spectra and ellipticity values at 208 and 280 nm, these last being diagnostic of helical polypeptides (Greenfield & Fasman, 1969; Johnson, 1990). It has been estimated that the four models proteins when dissolved in 50% TFE acquire a conformational state characterized by 45-60% helicity, compared with the 20-40% helical content in aqueous buffer (Fontana et al., 1995; Polverino de Laureto et al., 1995b,c; 1997; Galat, 1985; see also Shiraki et al., 1995). Conversely, the tertiary structure of lysozyme, as well as of the other model proteins, dissolved in 50% aqueous TFE is largely eliminated, as given by the strong reduction of the CD signal in the 250-300 nm region of the native protein (Strickland, 1974) (see Fig. 1). Thus, from the CD data, it can be inferred that the TFE-state of proteins is characterized by a high helical secondary structure, but lacking the specific tertiary interactions of the native protein.

Fig. 1 shows the CD spectra of thermolysin dissolved in 50% TFE at neutral pH. Clearly, thermolysin under these solvent conditions appears to maintain the integrity of its secondary and tertiary structure, since far- and near-UV CD spectra in buffer only and in 50% TFE are essentially identical. The unusual TFE-stability of thermolysin is in line with the fact that this exceptionally stable thermophilic enzyme can be crystallized from a 70% aqueous dimethylsulfoxide solution (Colman et al., 1974).

3. PROTEOLYSIS OF PROTEINS IN THEIR TFE-STATE

Four model proteins have been reacted with thermolysin in 50% TFE at neutral pH and 20-52°C for several hours. Calcium (5-10 mM) was added to the reaction mixture, since this ion stabilizes thermolysin (Roche & Voordouw, 1978; Fontana et al., 1977). While all four model proteins (see above) were relatively resistant to proteolysis by thermolysin if incubated for a short time in buffer only and without TFE, proteolysis of these proteins in their TFE-state occurred slowly and very selective, as evidenced from the analysis of aliquots of the proteolytic mixture by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) and reverse-phase high

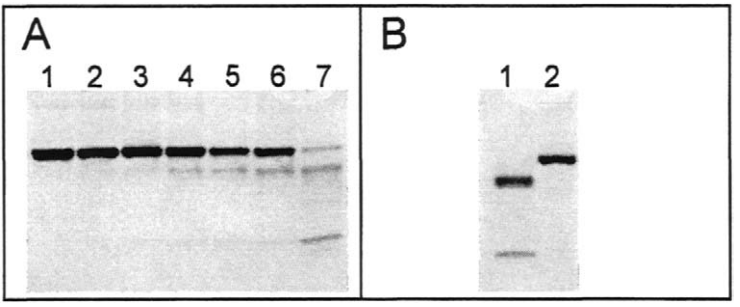

Figure 2. SDS-PAGE analysis of the proteolysis of lysozyme by thermolysin. (A) Proteolysis was conducted at 40°C in 50 mM Tris-HCl buffer, pH 7.0, containing 5 mM $CaCl_2$ in the presence of 50% TFE. The thermolysin:lysozyme ratio was 1:20 (by mass). Aliquots were taken from the reaction mixture after 0, 1, 2, 3, 4, 6 and 24 hours (lanes 1-7) and analyzed by SDS-PAGE under reducing conditions (Schägger & von Jagow, 1987). (B) SDS-PAGE analysis of purified nicked (lane 1) and intact (lane 2) lysozyme. The nicked protein species is constituted by fragments 1-97 and 98-129 covalently linked by disulfide bonds.

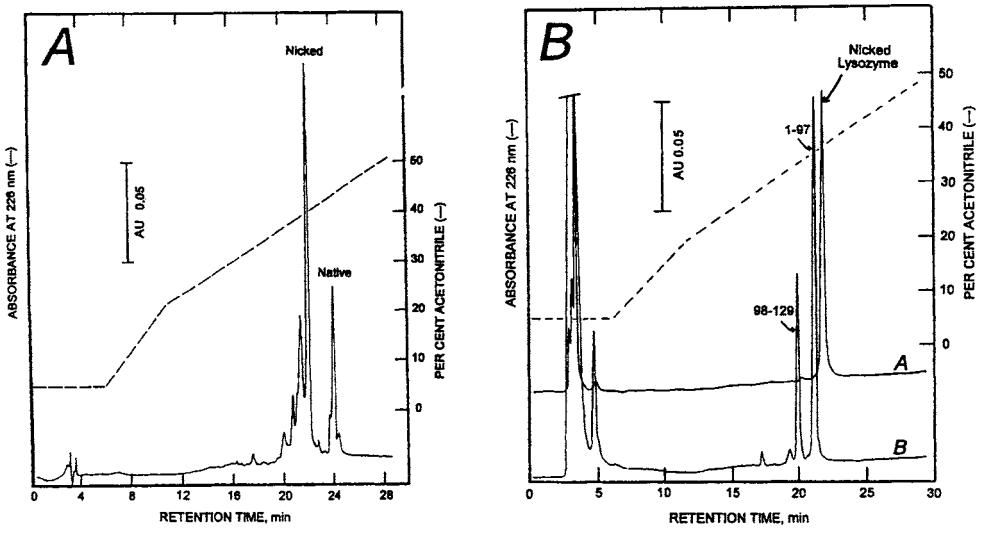

Figure 3. Reverse-phase HPLC analysis of the proteolytic mixture of thermolytic digest of lysozyme. (A) The sample of digested protein was produced by proteolysis for 5 hours at 52°C under the experimental conditions described in the legend to Fig. 2. An aliquot (~10 μg protein) of the proteolytic mixture was analyzed on a Vydac C4 column (4.6 × 150 mm) eluted with a gradient of acetonitrile in 0.05% of trifluoroacetic acid. (B) Reverse-phase HPLC analysis of purified nicked lysozyme (profile A) and of a sample of the same nicked protein after reduction and S-carboxamidomethylation (profile B). Reduction of the four disulfide bonds of the protein using dithiothreitol and subsequent S-alkylation with iodoacetamide produced the individual fragments 1-97 and 98-129.

Table 1. Limited proteolysis of proteins by thermolysin in 50% aqueous trifluoroethanol.[a]

Protein	E:S[b] (by mass)	Temperature (°C)	Peptide bond hydrolyzed[c]		Amino acid residues[d]		
			Fast	Slow	Ile	Leu	Phe
Cytochrome c	1:50	25	Gly^{56}-Ile^{57}	Gly^{45}-Phe^{46} Met^{80}-Ile^{81}	6	6	4
α-Lactalbumin	1:20	40	Ala^{40}-Ile^{41}	Gln^{2}-Leu^{3} Phe^{80}-Leu^{81}	8	13	4
Lysozyme	1:20	52	Lys^{97}-Ile^{98}	Ser^{24}-Leu^{25} Asn^{37}-Phe^{38}	6	8	3
Ribonuclease A	3:100	42	Asn^{34}-Leu^{35}	Thr^{45}-Phe^{46}	3	2	3

[a]The protein was dissolved (~ 1 mg/ml) in 50 mM Tris-HCl buffer, pH 7.0, containing 5 mM $CaCl_2$ in the presence of 50% TFE. In the case of cytochrome c, the buffer was 20 mM Tris, pH 7.8, containing 10 mM $CaCl_2$. An aliquot of thermolysin, dissolved in the same buffer, was added to the protein solution and then the reaction mixture was kept at the indicated temperature for 0.5-24 hours. The time course of the proteolysis was determined by SDS-PAGE and reverse-phase HPLC. [b]Thermolysin to protein ratio. [c]The sites of proteolytic cleavage along the polypeptide chain of the protein were determined by N-terminal sequencing of the various fragments produced and comparing these data with the known amino acid sequence of the protein. The term fast and slow refers to the initial (after 1-3 hours) and subsequent (after 6-24 hours) peptide bond fission, respectively. [d]Residues per molecule.

performance liquid chromatography (HPLC). As an example, the SDS-PAGE data obtained with lysozyme are shown in Fig. 2. It is seen that at 40°C lysozyme is slowly digested (1-6 hours of reaction) by thermolysin to two protein fragments only, while on prolonged reaction (24 hours) other fragments do appear in the Coomassie-stained gel. The thermolytic mixture of lysozyme (5 hours of reaction at 52°C) has been analyzed also by reverse-phase HPLC, allowing us to isolate to homogeneity the major protein species (nicked protein) (Fig. 3A). A sample of nicked lysozyme, after reduction with excess thiol and S-carboxamidomethylation, eluted from the reverse-phase HPLC column in two chromatographic peaks (Fig. 3B). The protein material of these two peaks was further analyzed in terms of N-terminal sequence and amino acid composition. These data, when compared with the known amino acid sequence of hen lysozyme, allowed us to establish that nicked lysozyme resulted from specific cleavage of the Lys97-Ile98 peptide bond and thus constituted by fragments 1-97 and 98-129 covalently linked by the four disulfide bridges of the protein. Moreover, it was found that upon prolonged proteolysis of lysozyme at high temperature, such as 24 hours at 40°C or 6 hours at 60°C, additional but few cleavages of the protein do occur. In the case of lysozyme, these minor cleavages occurred at the Ser24-Leu25 and Asn37-Phe38 peptide bonds (Polverino de Laureto et al., 1995c).

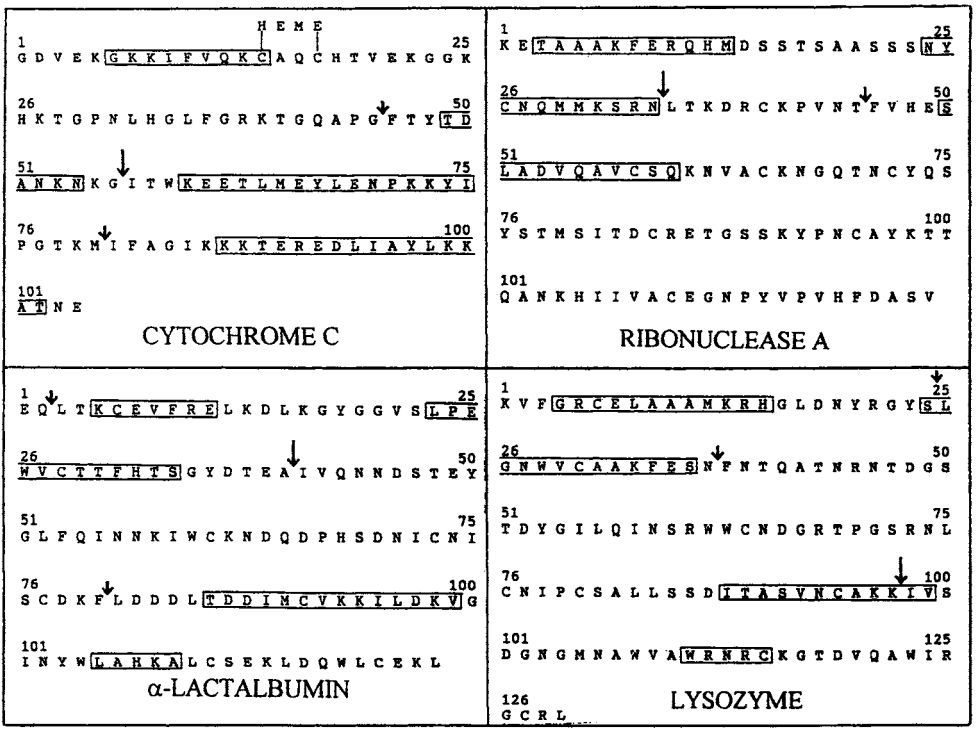

Figure 4. Sites of limited proteolysis along the amino acid sequences of horse cytochrome c, bovine pancreatic ribonuclease A, bovine α-lactalbumin and hen egg-white lysozyme. Major and minor arrows indicate the sites of fast and slow peptide bond fission, respectively (see also Table 1). The helical chain segments of the four proteins in their native state in aqueous buffer (without TFE), as given by crystallographic analysis of these proteins, are boxed.

The selective proteolysis of lysozyme in its TFE-state by thermolysin (Polverino de Laureto et al., 1995c) parallels those observed with bovine α-lactalbumin (Polverino de Laureto et al., 1995b), horse cytochrome c (Fontana et al., 1995) and ribonuclease A (Polverino de Laureto et al., 1997). The results of the proteolysis experiments on the four model proteins are summarized in Table 1 and Fig. 4. Of interest, it was possible to isolate to homogeneity a nicked protein constituted by two fragments covalently linked by disulfide bridges in the case of lysozyme, α-lactalbumin and ribonuclease A. The conformational, stability and functional properties of these nicked proteins were analyzed (Polverino de Laureto et al., 1995b,c; 1997). In the case of horse cytochrome c, the major thermolytic cleavage occurs at peptide bond Gly56-Ile57 (Fontana et al., 1995). The N-terminal fragment 1-56 maintains covalently bound the heme group of cytochrome c via a thioether linkage at the level of Cys14 and Cys17 and forms in

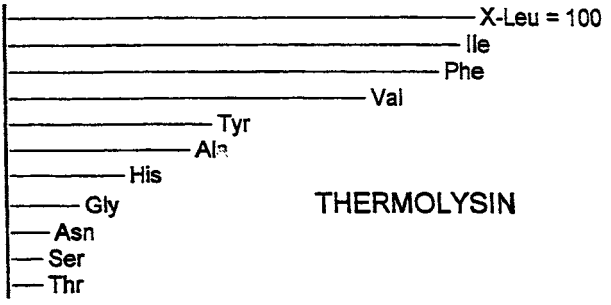

Figure 5. Relative rates of peptide bond hydrolysis by thermolysin in aqueous buffer at neutral pH. The rates are graphically expressed as percent of that of the hydrolysis of the peptide bond most preferentially cleaved by thermolysin. Of note, this metallo-endopeptidase cleaves at the amino side of bulky and hydrophobic amino acid residues (*e.g.*, the peptide bond Xaa-Leu is cleaved). The data are taken from Keil (1982).

aqueous buffer a non-covalent complex with the C-terminal fragment 57-104 (A. Fontana et al., unpublished).

4. DISCUSSION

The results of this study show that a protein dissolved in aqueous TFE can be cleaved by thermolysin at very few peptide bonds, leading to quite large protein fragments and/or nicked protein species rather resistant to further proteolysis. The ability of proteins in their helical TFE-state to resist extensive proteolytic degradation appears to be remarkable, if one considers the broad substrate specificity of thermolysin in aqueous buffer at neutral pH (Morihara & Tzusuki, 1970; Heinrikson, 1977; Keil, 1982). As shown in Fig. 5, thermolysin in water cleaves mostly at the amino side of bulky and hydrophobic amino acids, but significant hydrolysis occurs at other residues as well. On the other hand, the data given in Table 1 and Fig. 4 indicate that in aqueous TFE both major and minor cleavages by thermolysin of the four model proteins herewith investigated occur at peptide bonds involving only Ile, Leu and Phe residues. Nevertheless, even if the model proteins contain many Leu, Ile and Phe residues (see Fig. 4), thermolysin cleaves these proteins at very few sites only. With each model protein examined there is a peptide bond being cleaved first by thermolysin, followed by much slower cleavages at few additional peptide bonds.

The selective cleavage of proteins by thermolysin in aqueous TFE must be dictated both by the conformational state of the protein substrate and by some specific features of the biocatalyst dissolved in aqueous TFE. First of all, the protein substrate acquires a high helical state in TFE, since this alcohol appears to favour the formation of intramolecular hydrogen bonds of the α-helical conformations rather than hydrogen bonds with the solvent (Thomas & Dill, 1993; Jasanoff & Fersht, 1994; Cammers-Goodwin et al., 1996; Luo & Baldwin, 1997; Myers et al., 1998). Dobson and co-

workers (Buck et al., 1993, 1995, 1996; Alexandrescu et al., 1994; Affleck et al., 1994) demonstrated that at least the helical segments of the native protein are maintained in the TFE-state, even if hydrogen exchange measurements indicate that these helices are highly dynamic entities (fluctuating). Moreover, TFE also induces the formation of some extra helices which do not correspond to native-like helices (Buck et al., 1993, 1995, 1996; Alexandrescu et al., 1994). Thus, the high helical state of the protein substrate in aqueous TFE is not expected to be easily hydrolyzed by the protease, since a folded protein molecule is much more resistant to proteolysis than a random-coil polypeptide chain (Mihalyi, 1978). That the helical TFE-state of proteins is hampering extensive proteolysis is also in line with our proposal that proteolysis does not occur at the level of helical chain segments and that only flexible loops are the sites of fast proteolysis (Fontana et al., 1986, 1993, 1997a,b; Polverino de Laureto et al., 1995a).

The selective proteolysis of proteins in aqueous TFE by thermolysin occurs rather slowly, if one considers that proteolysis requires several hours of incubation at moderately high temperatures (*e.g.*, 25-52°C, see Table 1). This is due not only to the structured (helical) protein substrate hampering binding and adaptation at the active site of the protease, but also to the fact that the organic solvent causes significant enzyme inhibition (see Klibanov, 1997, for a recent discussion). It is tempting to speculate that, in the presence of the organic cosolvent, thermolysin can acquire an enhanced (overall and local) protein rigidity (Affleck et al., 1992a,b; Hartsough & Merz, 1992) causing a reduction of its catalytic potency, since some chain mobility is required for catalysis (Welch, 1986; Fersht, 1985; Kraut, 1988). The fact that thermolysin is much less active in the presence of TFE is also explained by the fact that proteases, including thermolysin (Wayne & Fruton, 1983), in the presence of organic cosolvents catalyze the reverse reaction, *i.e.* the synthesis instead of the hydrolysis of the peptide bond (Chaiken, 1981; Kullman, 1987).

The proteolysis of proteins in their TFE-state can be interpreted also on the basis of some features of the structure and dynamics of the native protein in aqueous solution, implying that the helical TFE-state appears to be related to the native state. First of all, as shown in Fig. 4, the sites of initial cleavages within the polypeptide chains of the four model proteins occur outside the helical chain segments of the native proteins. Accepting our view that helices are quite rigid rods not prone to proteolysis (Fontana et al., 1986, 1993, 1997a,b; Polverino de Laureto et al., 1995a), proteins in their TFE-state appear to maintain at least the helical chain segments of their native state. Moreover, the sites of the proteolytic cleavage along the protein chain (Fig. 4) should be the most flexible ones, since we have previously demonstrated that there is a correlation between sites of limited proteolysis and sites of higher segmental mobility (Fontana et al., 1986). For example, the chain region encompassing the Asn34-Leu35 peptide bond which is cleaved in ribonuclease A in its TFE-state (see Fig. 4) is the most flexible one also in the native protein in aqueous buffer, as given by hydrogen exchange measurements (Kiefhaber & Baldwin, 1995) and by the fact that this region is poorly defined in the X-ray density map (Wlodawer et al., 1982). Of interest, the same Asn34-Leu35 peptide bond is the site of initial hydrolysis when ribonuclease is reacted with thermolysin on

mild heating in aqueous buffer (Arnold et al., 1996). All these data therefore indicate that the chain region encompassing the Asn34-Leu35 peptide bond in ribonuclease A displays higher flexibility than the rest of the protein chain both in the native state and in TFE-state, implying some similarity of structure and dynamics between the two states.

In summary, proteolysis of proteins in aqueous TFE by thermolysin is a novel and useful procedure for the selective enzymatic fragmentation of proteins. The technique is simple to use, modest in demands for protein sample and experimental effort and will find useful applications in protein structure research for the preparation of rather large protein fragments for sequencing purposes by the Edman technique and for biophysical and functional studies.

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