Probing the Molten Globule State of α-Lactalbumin by Limited Proteolysis[†]

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ABSTRACT: Limited proteolysis has been used to probe the partially folded state of bovine α-lactalbumin (BLA) at acid pH (A-state) or dissolved in aqueous trifluoroethanol (TFE-state). The sites of proteolytic fission have been determined by isolation of the various BLA fragments and comparison of their N-terminal amino acid sequence and amino acid composition after acid hydrolysis, as well as their molecular mass determined by mass spectrometry, with the known sequence of BLA. Incubation of BLA with pepsin at 20-22 °C and pH 2.0 in the presence of 0.1 M NaCl results in very rapid cleavage of the 123-residue chain at peptide bond Ala40-Ile41 and subsequently at Leu52-Phe53, leading to a nicked species of BLA constituted by the two fragments 1-40 and 53-123 cross-linked by the four disulfide bridges of the protein. Much slower proteolytic cleavage occurs at Tyr103-Trp104. The highly helical conformational state acquired by BLA when dissolved in aqueous buffer (pH 7.0) containing 50% (v/v) TFE was probed by the TFE-resistant thermolysin. Proteolytic cleavage occurs at the peptide bond Ala40-Ile41 and much more slowly at Phe80-Leu81. Moreover, the peptide bond Gln2-Leu3 at the N-terminus of the chain is partially cleaved by thermolysin. Conversely, native BLA in a pH 7.0 buffer is rather resistant to proteolysis. Considering the broad substrate specificity of both pepsin and thermolysin and, thus, the very numerous potential sites of proteolytic attack along the 123-residue chain of BLA, these results indicate that BLA in its partially folded A- or TFE-state is a more dynamic entity than in the native state, but is still a structured and relatively rigid protein, preventing extensive degradation by proteolysis. All sites of limited proteolysis observed in this study are located outside the helical chain segments of BLA in its native or partially folded states [Alexandrescu, A. T., Ng, Y.-L., & Dobson, C. M. (1994) *J. Mol. Biol. 235*, 587-599]. The fast initial nicking at chain segment 40-53 of BLA by both pepsin and thermolysin indicates that this region in both the A- and TFE-states of BLA are characterized by the exposure and flexibility required for a productive interaction at the active site of the attacking protease. Indeed, previous NMR measurements have indicated that the single β-sheet region (chain segment 40-53) of the native structure, at variance from the helical segments, is not presented in the A- or TFE-state of BLA.

The detailed mechanism by which the amino acid sequence of a protein directs the folding to its biologically active, three-dimensional structure is a fundamental problem in biophysical chemistry that is not yet fully understood (Creighton, 1992). It is generally accepted now that the folding of a polypeptide chain proceeds via folding intermediates, thus directing the folding through a limited number of pathways and consequently leading to the final folded state within a reasonably short time (Levinthal, 1968; Kim & Baldwin, 1982, 1990). Thus, in order to possibly formulate mechanisms for protein folding, a detailed knowledge of the structure, stability, and dynamics of folding intermediates is needed. However, the intermediates often exist only transiently in the course of folding, so that their reliable identification and detailed structural characterization are usually difficult if not possible.

A protein species that appears to possess the properties of a kinetic folding intermediate is the "molten globule state", which can be generated from a globular protein under specific and mildly denaturant solvent conditions and occurs at equilibrium, thus allowing analysis of its structural features (Ptitsyn, 1987, 1992). The key characteristics of a molten globule state are a well-defined and native-like secondary structure, lack of specific tertiary interactions, and a more expanded and flexible structure with respect to that of the native protein (Dolgikh *et al.*, 1981; Ohgushi & Wada, 1983; Kuwajima, 1989; Baum *et al.*, 1989; Christensen & Pain, 1991; Dobson, 1992; Haynie & Freire, 1993). Recent studies have shown that the molten globule state is indeed a relevant and perhaps universal species in the pathway of protein folding (Ptitsyn, 1992). It is of considerable interest, therefore, to generate and characterize partially folded protein species occurring at equilibrium.

The most extensively characterized molten globule state is the one obtained by acid-induced unfolding of α-lactalbumin at pH 2.0 (A-state).[1] These studies took advantage of the knowledge of the detailed three-dimensional structure

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[1] Abbreviations: BLA, bovine α-lactalbumin; CAM-BLA, reduced and *S*-carboxamidomethylated BLA; TFE, trifluoroethanol; CD, circular dichroism; [θ], mean residue ellipticity; Tris, tris(hydroxymethyl)-aminomethane; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography; PTH, phenylthiohydantoin; PTC, phenylthiocarbamoyl; TFA, trifluoroacetic acid; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; Chaps, 3-(cyclohexylamino)-1-propanesulfonic acid; NMR, nuclear magnetic resonance; A- and TFE-states, partially unfolded states of BLA induced by acid (pH 2.0) or 50% (v/v) aqueous TFE (pH 7.0).

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of the baboon α-lactalbumin at 1.7 Å resolution (Acharya *et al.*, 1989, 1991). A great number of different techniques and approaches have been used over the years to characterize this A-state, which can be considered a prototype molten globule. Intrinsic viscosity (Dolgikh *et al.*, 1985), quasielastic light scattering (Gast *et al.*, 1986), circular dichroism (Dolgikh *et al.*, 1981; Kuwajima *et al.*, 1985), and differential calorimetry (Xie *et al.*, 1991; Yutani *et al.*, 1992; Griko *et al.*, 1994), as well as binding of the hydrophobic probe ANS (Semisotnov *et al.*, 1991) and disulfide rearrangement experiments (Ewbank & Creighton, 1991; Creighton & Ewbank, 1994), allowed the discovery of much detailed knowledge of the A-states of human, bovine, and guinea pig α-lactalbumin. Whereas these techniques gave a global picture of a molten globule, more recently NMR spectroscopy was used for structural characterization of the A-state of α-lactalbumin at the atomic level (Baum *et al.*, 1989; Chyan *et al.*, 1993; Alexandrescu *et al.*, 1993), providing clear-cut evidence that the A-state is a dynamic conformational state, but nevertheless possesses stable regions of localized and native-like helical secondary structure and largely disordered tertiary structure. Recently, a trifluoroethanol (TFE)-induced, partially folded state of α-lactalbumin was carefully analyzed by circular dichroism (CD) and NMR spectroscopy, showing that the TFE-state of α-lactalbumin (at pH 2.0) possesses a high content of helical secondary structure of native-like characteristics, whereas the single β-sheet of the native protein appears to be largely eliminated (Alexandrescu *et al.*, 1994). Moreover, the helical domain of human α-lactalbumin has been produced by genetic engineering methods and shown to form, in isolation, a molten globule with the same overall tertiary fold as that found in the intact protein (Peng & Kim, 1994).

The present study was undertaken with the view to contribute to the analysis of a protein molten globule by using proteolytic enzymes as probes of protein structure and dynamics (Mihalyi, 1978; Neurath, 1980; Wilson, 1991). In previous studies, we have shown that limited proteolysis (Lindenström-Lang, 1949) of a globular protein, in its native state, occurs only at exposed and flexible loops and never at helical chain segments (Fontana *et al.*, 1986; Signor *et al.*, 1990; Polverino de Laureto *et al.*, 1994, 1995). This conclusion was reached on the basis of our own experimental work, as well as by detailed analysis of the results of limited proteolysis experiments conducted on a number of globular proteins of known three-dimensional structure (Fontana *et al.*, 1993). To probe the structures of the A- and TFE-states of bovine α-lactalbumin (BLA) in acid or in the presence of aqueous TFE, we made use of pepsin and thermolysin, respectively. These proteases have the advantage of being stable and active in acid and at neutral pH in the presence of aqueous organic solvents (Welinder, 1988; Kitaguchi & Klibanov, 1989), respectively. Moreover, both proteases show broad substrate specificity, with pepsin cleaving at the C-terminus of mainly bulky and hydrophobic amino acid residues and thermolysin cleaving at the amino side of Leu, Ile, Phe, Tyr, and other residues as well (Heinrikson, 1977; Keil, 1982). Thus, it was anticipated that proteolytic cleavage would be dictated by the stereochemistry and dynamics of the BLA substrate and not by the specificity of the protease. Indeed, herewith it is shown that the molten globule state of BLA is sufficiently folded and relatively rigid, preventing its extensive proteolytic degradation and leading instead to quite selective peptide bond fissions. The results obtained are discussed on the basis of the structural and dynamic features of the partially folded species (A- and TFE-states) of BLA investigated by NMR measurements (Baum *et al.*, 1989; Alexandrescu *et al.*, 1993, 1994).

EXPERIMENTAL PROCEDURES

Proteins and Chemicals. Bovine α-lactalbumin (BLA), pepsin, and thermolysin were obtained from Sigma (St. Louis, MO). The BLA preparation was checked by reverse-phase high-performance liquid chromatography (HPLC), capillary electrophoresis in 0.1 M phosphoric acid (pH 2.5), and sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) and shown to be at least 90% pure. A sample of reduced and *S*-carboxamidomethylated BLA (CAM-BLA) was prepared by reduction of the four disulfide bonds of the protein with dithiothreitol in 0.5 M Tris-HCl buffer (pH 8.0) containing 6 M guanidinium chloride and 2 mM EDTA. The reaction mixture was kept at 37 °C for 2 h under nitrogen atmosphere and then 50 equiv of iodoacetamide was added to the reaction mixture after 30 min under nitrogen atmosphere in the dark. CAM-BLA was purified by reverse-phase HPLC on a Vydac C_4 column using an acetonitrile/water gradient.

The reagents and solvents used for SDS−PAGE were purchased from Bio-Rad (Richmond, CA), those for peptide/protein sequence analysis were from Applied Biosystems (Foster City, CA), and those for amino acid analysis were from Waters (Milford, MA). Trifluoroethanol (TFE), trifluoroacetic acid (TFA), tris(hydroxymethyl)aminomethane (Tris), *N*-[2-hydroxy-1,1-bis(hydroxymethyl)aminomethane] (Tricine), 3-(cyclohexylamino)-1-propanesulfonic acid (Chaps), and cyanogen bromide (CNBr) were obtained from Fluka (Basel, Switzerland). All organic solvents were of analytical grade purchased from C. Erba (Milan, Italy) or Merck (Darmstadt, Germany).

Electrophoresis. SDS−PAGE was carried out in a vertical slab gel apparatus (Miniprotean-II, Bio-Rad) using the Tricine buffer system (Schägger & von Jagow, 1987). The gels were stained with Coomassie Brilliant Blue R-250. Mixtures of protein fragments of known molecular mass were used as calibration standards. A partial CNBr digest of BLA at the level of Met90, producing fragments 1−90 and 91−123, was routinely used. Electroblotting (Matsudaira, 1987) of proteins and protein fragments from SDS−PAGE gels onto the Pro-Blott membrane (Applied Biosystems) was conducted by using the Trans-Blott system (Bio-Rad) in 10 mM Chaps (pH 11) containing 10% methanol for 1 h at 75 V, and then the Pro-Blott membrane was stained with Coomassie Blue. Stained bands were excised from the membrane with a razor and subjected to protein sequence analysis.

High-Performance Liquid Chromatography. Reverse-phase HPLC was performed on a Waters-Millipore instrument (Model 510) connected to a detector (Model 484). A Vydac C_4 column (4.6 × 150 mm) purchased from The Separations Group (Hesperia, CA) was utilized for the analysis of proteolytic mixtures and for the isolation of nicked species and fragments of BLA. The column was eluted with a gradient of water/acetonitrile containing 0.05% TFA.

Amino Acid Analysis and Sequencing. Protein/peptide samples (50−300 pmol), contained in heat-treated borosilicate tubes (4 × 50 mm), were hydrolyzed *in vacuo* for 1 h

at 150 °C with 0.2 mL of 6 N HCl containing 0.1% (by weight) phenol on the Pico-Tag workstation (Waters). The amino acids were derivatized with phenylisothiocyanate (Heinrikson & Meredith, 1984), and the resulting phenylthiocarbamoyl (PTC) derivatives were analyzed by HPLC using the Pico-Tag C_{18} column (4.6 × 150 mm) (Waters).

N-Terminal sequence analysis was performed with an Applied Biosystems pulsed liquid-phase sequencer (Model 477A) equipped with an on-line analyzer (Model 120A) of phenylthiohydantoin (PTH) derivatives of amino acids. Standard manufacturer's procedures and programs were used with minor modifications.

Mass Determination. Mass spectra were recorded using a MALDI-TOF mass spectrometer (Reflex, Bruker). A mixture of 5 μL of 10^{-5} M analyte solution and 5 μL of 10^{-1} M sinapinic acid (Sigma) in 20% acetonitrile was applied to the metallic probe tip and dried *in vacuo*. Then a pulsed laser beam (337 nm, 3 ns) was focused on the sample, and the ions were accelerated with a linear potential of 15 kV. Mass calibration was performed with horse heart myoglobin (average molecular mass = 16 950.5 Da) and bovine insulin (average molecular mass = 5733.6 Da) utilized as external standards. Raw data were analyzed by X-Mass software provided by Bruker and are reported as average mass. Alternatively, protein samples were analyzed by electrospray mass spectrometry using a VG BIO-Q triple-quadrupole mass spectrometer equipped with an electrospray source. Mass calibration was performed by means of the multiply charged ions from a separate injection of horse myoglobin. Masses are reported as average mass.

Circular Dichroism Measurements. Circular dichroism (CD) spectra of BLA in the far- and near-ultraviolet regions were recorded on a Jasco J-710 spectropolarimeter equipped with a thermostated cell holder. The temperature of the sample solution in the cuvette was controlled with a Neslab RTE-100 water bath. All spectra were smoothed and baseline corrected. Cells of path length 1 and 5 mm in the far- and near-ultraviolet regions, respectively, were employed. For calculation of the mean residue ellipticity, $[\theta]$, a mean residue weight of 115 was used, as calculated from the amino acid composition of BLA. The protein concentration was determined spectrophotometrically using molar extinction at 280 nm of $E^{1\%} = 20.1$ (Edelhoch, 1967). The CD spectra in the far- and near-ultraviolet regions were measured at a protein concentrations of 0.1 and 0.45 mg/mL, respectively. Mean residue ellipticity, $[\theta]$, is expressed in deg cm^2 dmol^{-1} and was calculated from the formula $[\theta] = \theta(10cnl)$, where θ is the ellipticity observed (mdeg), c is the protein concentration (mol/L), l is the path length of the cuvette (cm), and n is the number of amino acid residues of BLA (123).

Proteolysis of BLA with Pepsin. Digestion of BLA dissolved (1 mg/mL) in 10 mM HCl/0.1 M NaCl (pH 2.0) was performed at 20−22 °C utilizing pepsin at a protease:substrate ratio from 1:100 to 1:2000 (by weight). At intervals, aliquots (5 μL) were removed from the mixture and the proteolysis was stopped by adding a 0.3% ammonia (v/v) solution (10 μL). The samples were concentrated using the Speed-Vac system of Savant (Farmingdale, CT) and then dissolved with the SDS−PAGE solubilization buffer [20 mM Tris-HCl (pH 6.8) containing 2.5% SDS, 0.02% bromophenol blue, and 5% β-mercaptoethanol]. The samples were heated for 5 min in boiling water and subjected to slab gel SDS−PAGE analysis. Aliquots of the digestion mixture were also analyzed by reverse-phase HPLC using a Vydac C_4 column eluted with a gradient of acetonitrile in 0.05% aqueous TFA.

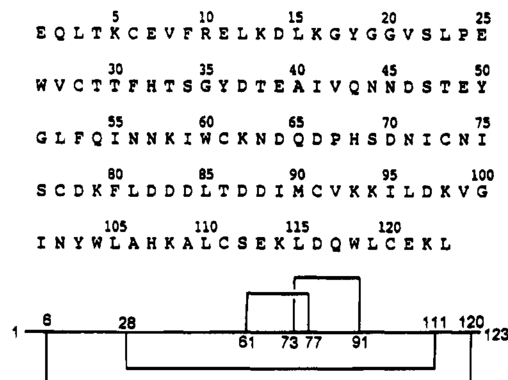

FIGURE 1: Amino acid sequence of bovine α-lactalbumin. The diagram at the bottom indicates the location of the four internal disulfide bridges of the protein.

Proteolysis of BLA with Thermolysin. BLA was dissolved (1 mg/mL) in 50 mM Tris-HCl buffer (pH 7.0) containing 5 mM $CaCl_2$ and varying concentrations of TFE (from 0 to 50%, by volume) and treated with thermolysin at a protease:substrate ratio of 1:20 (by weight). Proteolysis was stopped by taking aliquots (10 μL) from the mixture and mixing with 5% (v/v) aqueous TFA (50 μL). The samples were concentrated in the Speed-Vac and then analyzed by SDS−PAGE and reverse-phase HPLC.

Purification and Identification of BLA Fragments. The identity of the proteolytic fragments of BLA was established on the basis of their apparent molecular mass given by SDS−PAGE analyses, N-terminal sequencing of peptide/protein bands electroblotted from SDS−PAGE gels onto the ProBlott membrane, and amino acid analysis after acid hydrolysis of samples of fragments or nicked species of BLA purified by micropreparative reverse-phase HPLC. Moreover, the exact mass of the peptide/protein species was also evaluated by MALDI-TOF or electrospray mass spectrometry. Correlation of the results of these various analyses with the amino acid sequence of BLA (Figure 1) allowed identification of the proteolytic fragments and, thus, determination of the sites of proteolytic cleavage of BLA.

RESULTS

Circular Dichroism Measurements. CD measurements have been used previously to characterize the various conformational states of bovine, human, and guinea pig α-lactalbumin (Dolgikh *et al.*, 1981; Ikeguchi & Sugai, 1992; Kuwajima *et al.*, 1985; Alexandrescu *et al.*, 1994). In order to characterize the conformational properties of BLA when dissolved under the specific experimental conditions employed for proteolysis (see the following), CD spectra of BLA were measured in acid solution and in the presence of TFE in order to generate the A- (Kuwajima *et al.*, 1985) and TFE-states (Alexandrescu *et al.*, 1994). The far- and near-ultraviolet CD spectra of BLA in 10 mM HCl/0.1 M NaCl (pH 2.0) favorably compare with those previously reported (Kuwajima *et al.*, 1985) and show that the A-state BLA largely maintains the secondary structure of the native protein, but lacks specific tertiary interactions. Of note, curve fitting of the CD spectrum in the far ultraviolet allowed Kuwajima *et al.* (1985) to determine that the A-state roughly contains a similar percentage of α-(22−33%) and β-structure (20−31%), depending upon the computational method employed (Greenfield & Fasman, 1969; Yang *et al.*, 1986),

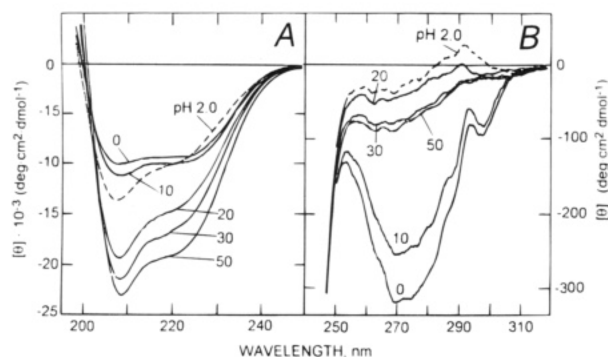

FIGURE 2: Circular dichroism spectra of BLA in acid or in the presence of aqueous TFE. Spectra were recorded at room temperature at protein concentrations of 0.1 and 0.45 mg/mL in the far- and near-ultraviolet regions, respectively. The solid lines correspond to spectra obtained in 50 mM Tris-HCl buffer (pH 7.0) containing 0, 10, 20, 30, and 50% (v/v) TFE. The CD spectrum of BLA (dashed line) in acid was measured in 10 mM HCl/0.1 M NaCl, pH 2.0.

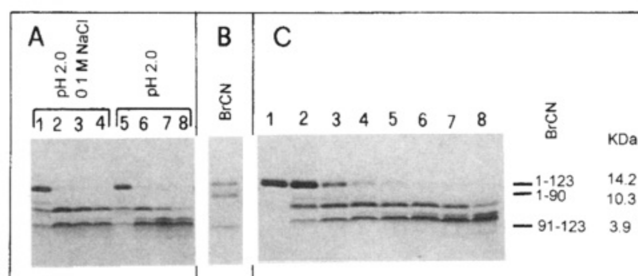

FIGURE 3: Limited proteolysis of BLA by pepsin monitored by SDS−PAGE. (A) Digestion was conducted at pH 2.0 and 20−22 °C in the presence or absence of 0.1 M NaCl. The proteolysis was conducted by utilizing an E:S ratio of 1:750 (by weight) (see Experimental Procedures). Samples (~5 μg) were taken from the reaction mixture after 5 (1, 5), 25 (2, 6), 45 (3, 7), and 60 min (4, 8) and dissolved in the sample buffer of the electrophoresis system (Schägger & von Jagow, 1987). (B) Partial CNBr digest of BLA containing the intact protein (14.2 kDa), fragment 1−90 (10.3 kDa), and fragment 91−123 (3.9 kDa). (C) Time course of the proteolysis of BLA by pepsin after 0 (1), 5 (2), 15 (3), 25 (4), 35 (5), 45 (6), 60 (7), and 90 min (8).

similar to that of the native state. The CD spectra of BLA in aqueous buffer (pH 7.0) in the presence of increasing concentrations of TFE provide evidence that TFE induces a conformational transition to a state characterized by a high content of helical secondary structure, as deduced by the shape of the spectrum and ellipticity values at 208 and 220 nm (Greenfield & Fasman, 1969). In the presence of 50% (v/v) TFE at pH 7.0, it can be estimated that the helix content of BLA is approximately 60%, compared with the 30% helical content of the native state. Conversely, the tertiary structure of BLA in its TFE-state is largely eliminated, as shown by the strong reduction in the CD signal in the 250−300 nm region of the native species (Figure 2B). Thus, it can be inferred that the TFE-state, as the A-state, lacks the specific tertiary interactions of the native protein. In 10−20% (v/v) TFE, it appears that BLA acquires conformational features that are intermediate with respect to those of the native and TFE-state in 50% (v/v) TFE.

Proteolysis with Pepsin. Several preliminary experiments of limited proteolysis of BLA in its A-state (pH 2.0) were carried out using pepsin at various substrate:proteolytic enzyme (E:S) ratios, temperatures, and times of incubation. The time course of the digestion was followed by SDS−PAGE analysis of aliquots taken from the proteolytic mixture. From the SDS−PAGE gels shown in Figure 3A, it is evident that pepsin leads to efficient but clean fragmentation of BLA, since very few protein fragments are produced at 20−22 °C utilizing an E:S ratio of 1:750 (by weight). At the initial stages of the digestion (5 min, see Figure 3A, lane 1), a faint band of approximate molecular mass 9.5 kDa is seen in the gel, which disappears later on since the corresponding protein fragment species is further digested to a species of lower electrophoretic mobility. After 1 h of reaction, two fragments of approximate molecular masses 8.6 and 4.2 kDa are the only species seen in the gel. A partial digest of BLA with CNBr cleaving at the single Met80 of the protein and leading to fragments 1−90 (10.3 kDa) and 91−123 (3.9 kDa) used as molecular mass markers (Figure 3B). Blotting and protein sequencing of the fragment species of 9.5 kDa seen in the SDS−PAGE gel after a 5 min reaction (see Figure 3A, lane 1) gave Ile-Val-Gln-Asn as the N-terminal amino acid sequence. Similarly, the sequences of the two main fragments of 8.6 and 4.2 kDa accumulating in the proteolytic mixture (Figure 3A, lanes 2−4) were Phe-Gln-Ile-Asn and Glu-Gln-Leu-Thr, respectively. Comparison of these sequences and molecular masses with the known amino acid sequence of BLA (Figure 1) allowed us to propose that the initial nicking of BLA by pepsin occurs at the Ala40−Ile41 peptide bond, followed by a rapid cleavage of the Leu53−Phe53 peptide bond, thus leading to fragments 1−40 and 53−123 as the most resistant species to further proteolysis under the specific experimental conditions (pH 2.0, 0.1 M NaCl). The SDS−PAGE data in Figure 3A (lanes 5−8) show that proteolysis by pepsin of BLA at pH 2.0 without salt is somewhat faster and that the 8.6 kDa band is further degraded to fragments of approximate molecular masses 6.5 and 3.0 kDa. These last fragment species were also produced from BLA in its A-state in the presence of 0.1 M NaCl upon prolonged proteolysis (see Figure 3C). The observed salt effect on the proteolysis is in keeping with the fact that the addition of salt enhances both the structure (Ikeguchi & Sugai, 1989) and stability (Griko *et al.*, 1994) of BLA, rendering the protein substrate more structured and rigid and thus less amenable to proteolysis.

In order to firmly establish the identity of the BLA fragments and thus the sites of proteolytic cleavage, an aliquot of the peptic digestion mixture (E:S = 1:750, 20−22 °C, 60 min) was applied to a Vydac C_4 column in order to purify the proteolytic fragments. The peptide/protein material eluted from the column (Figure 4) was analyzed for its amino acid composition after acid hydrolysis, N-terminal sequence, and molecular mass by mass spectrometry. The data given in Table 1 establish that the peptide bonds cleaved by pepsin are Ala40−Ile41 and Leu52−Phe53, followed by a cleavage at the Tyr103−Trp104 peptide bond. Thus, all fragment species corresponding to cleavages at these three peptide bonds have been isolated and characterized (1−40, 41−52, 43−103, and 104−123). Of note, fragments 1−40 and 104−123 (peak P2), as well as fragments 1−40 and 53−123 (peak P4), are covalently cross-linked by disulfide bridges (see Figure 1) and thus are eluted together from the reverse-phase HPLC column (Figure 4).

Some experiments of proteolysis by pepsin were performed by also utilizing a sample of reduced and *S*-carboxamidomethylated BLA (CAM-BLA). It was found that the proteolysis was somewhat faster, but the pattern of protein fragmentation qualitatively deduced from the SDS−PAGE gels was similar to that observed with intact BLA (not

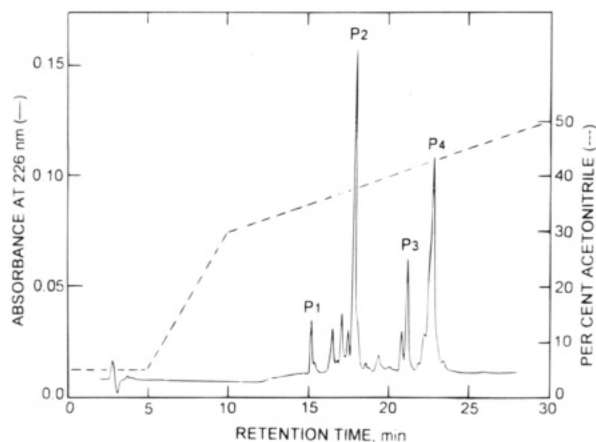

FIGURE 4: Reverse-phase HPLC analysis of the proteolysis mixture of BLA in its A-state by pepsin. The sample analyzed was obtained by proteolysis of BLA at 20−22 °C for 1 h with pepsin in 0.1 M NaCl (pH 2.0) at an E:S ratio of 1:750 (by weight). A Vydac C_4 column (4.6 × 150 mm) was employed. Elution was carried out at a flow rate of 0.6 mL/min with a gradient of acetonitrile in 0.05% TFA. The effluent was monitored at 226 nm.

shown). This is in line with previous conclusions, based on CD measurements, that the integrity of the four disulfide bonds of BLA is not critical for the formation of the A-state of the protein at pH 2.0 (Kuwajima, 1977; Ikeguchi & Sugai, 1989).

Proteolysis with Thermolysin. The proteolysis of BLA by thermolysin was studied in 50 mM Tris-HCl buffer (pH 7.0) containing 5 mM $CaCl_2$. Calcium was added to the reaction mixture, since this ion stabilizes both BLA (Segawa & Sugai, 1983) and thermolysin (Roche & Voordouw, 1978; Fontana *et al.*, 1977). In the absence of TFE, BLA was found to be resistant to proteolysis by thermolysin when reacted for 2 h at 20−22 °C at an E:S ratio of 1:20 (by weight); BLA was recovered unchanged from a Vydac C_4 column. On the other hand, when proteolysis was conducted utilizing a sample of CAM-BLA, the protein was fully digested to small peptides (not shown).

Figure 5 shows the SDS−PAGE analysis of the proteolysis mixture of BLA digested with thermolysin (E:S = 1:20, by weight) in the presence of increasing concentrations of TFE (from 20 to 50%, v/v) (part A) and in 50% (v/v) TFE as a function of time of incubation (B) or at different temperatures (C). It is seen that in moderate concentrations of TFE (10−20%, v/v) thermolysin digestion of BLA leads to the two main fragments (see Figure 5A, lane 3) of electrophoretic mobility similar to that observed when BLA in its A-state is

Table 1: Analytical Characterization of Fragments of BLA Produced by Peptic Digestion[a]

Amino Acid Composition[b]				
amino acid	P1 (41−52)	P2 (1−40) (104−123)	P3 (53−103)	P4 (1−40) (53−123)
Asx	3.2 (3)	3.1 (3)	14.7 (16)	18.7 (19)
Thr	1.0 (1)	4.2 (5)	1.1 (1)	4.8 (6)
Ser	0.6 (1)	3.1 (3)	2.3 (2)	5.2 (5)
Glx	2.5 (2)	8.6 (9)	2.3 (2)	11.2 (11)
Pro		1.2 (1)	0.9 (1)	2.1 (2)
Gly	0.9 (1)	4.5 (4)	1.3 (1)	5.2 (5)
Ala		3.0 (3)		3.2 (3)
Val	0.5 (1)	2.9 (3)	1.7 92)	4.8 (5)
Met			1.1 (1)	1.5 (1)
Ile	0.5 (1)		6.0 (7)	6.5 (7)
Leu	1.2 (1)	8.3 (9)	2.8 (3)	12.3 (12)
Tyr	1.1 (1)	1.9 (2)	0.9 (1)	3.1 (3)
Phe		1.8 92)	1.7 (2)	3.8 (4)
Lys		5.3 (6)	5.0 (6)	11.3 (12)
His		2.2 (2)	1.1 (1)	3.6 (3)
Arg		1.1 (1)		0.9 (1)
Trp		nd (3)	nd (1)	nd (4)
1/2-Cys		nd (4)	nd (4)	nd (8)
no. of residues	12	60	51	111

N-Terminal Sequence[c]				
cycle no.	P1	P2	P3	P4
1	Ile(202)	Glu(414)Trp(379)	Phe(203)	Glu(184)/Phe(223)
2	Val(101)	Gln(438)/Leu(486)	Gln(115)	Gln(273)
3	Gln(49)	Leu(574)/Ala(474)	Ile(305)	Leu(208)/Ile(205)
4	Asn(53)	Thr(403)/His(236)	Asn(86)	Thr(129)/Asn(98)
5	Asn(67)	Lys(806)	Asn(85)	Lys(124)/Asn(161)

Molecular Mass[d]			
P1	P2	P3	P4
1354 (1352.38)	6905 (6907.72)	5957 (5958.65)	12890 (12890.42)

[a] The protein fragments, named P1−P4, have been isolated to homogeneity by micropreparative reverse-phase HPLC (see text and Figure 4 for details). [b] Amino acid compositions are reported as amino acid residues per molecule. Expected values are given in parentheses and were calculated from the amino acid squence of BLA. The values of Asx and Glx are the sum of Asp and of Glu and Gln calculated from the sequence of BLA. The figures given are average values obtained from three separate amino acid analyses. Determined by MALDI-TOF mass spectrometry (see Experimental Procedures). Calculated values are given in parentheses. no., number; nd, not determined. [c] Sequence analysis was performed on samples purified by reverse-phase HPLC (Figure 4). The results of automatic sequencing are reported as yields (in pmol) of phenylthiohydantoin (PTH) derivative of amino acid recovered on the sequencer at each cycle of Edman degradation. The results are reported for the first five cycles; even for some fragments clear-cut sequence data have been obtained for longer N-terminal sequences.

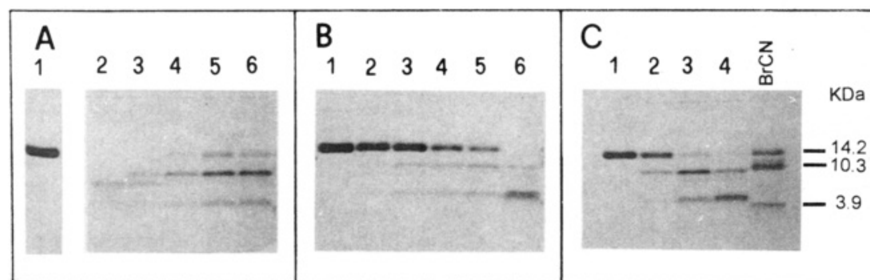

FIGURE 5: Limited proteolysis of BLA by thermolysin monitored by SDS−PAGE. Proteolysis of BLA was conducted in 50 mM Tris-HCl buffer (pH 7.0) containing 5 mM $CaCl_2$. All experiments were conducted utilizing an E:S ratio of 1:20 (by weight). (A) Effect of TFE concentration of the pattern of proteolytic fragments of BLA. Samples (~5 μg) were taken from the reaction mixture after 1 h at 20−22 °C. The reaction buffer contained 0 (1), 10 (2), 20 (3), 30 (4), 40 (5), and 50% (v/v) (6) TFE. (B) Time course of the proteolysis of BLA in 50% (v/v) TFE by thermolysin at 40 °C. The reaction was stopped after 0 (1), 1 (2), 2 (3), 4 (4), 6 (5), and 24 h (6). (C) Effect of temperature of incubation on the thermolytic digestion of BLA in 50% (v/v) TFE. The reaction was conducted for 6 h at 20 (2), 40 (3), and 50 °C (4). BrCN: a partial CNBr digest of BLA.

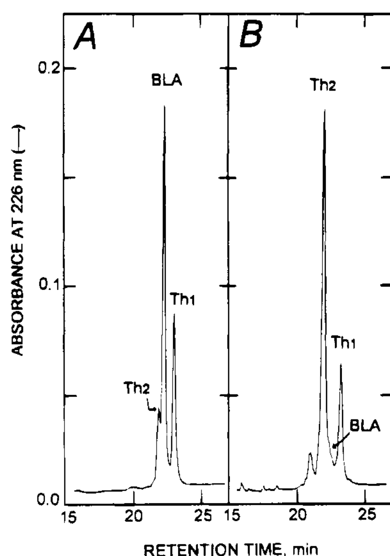

FIGURE 6: Reverse-phase HPLC analysis of the proteolytic mixture of BLA with thermolysin. Proteolysis was conducted with an E:S ratio of 1:20 (by weight) in 50 mM Tris-HCl buffer (pH 7.0) containing 5 mM $CaCl_2$ and 50% TFE (v/v) for 6 h at 22 (A) or 50 °C (B). Aliquots of the proteolytic mixtures were analyzed by reverse-phase HPLC utilizing a Vydac C_4 column (4.6 × 150 mm) and eluted at a flow rate of 0.6 mL/min, with a linear gradient of acetonitrile containing 0.05% (v/v) TFA from 5 to 30% in 4 min and from 30 to 50% in 18 min.

digested by pepsin (see above; Figure 3A). Since in this work we were interested in the TFE-state of BLA (Alexandrescu *et al.*, 1994), the proteolysis phenomenon in buffer (pH 7.0) containing 50% (v/v) TFE was studied in detail. The SDS−PAGE analysis of the time course of the digestion of BLA at 40 °C (Figure 5B) shows that there is a slow proteolysis leading to two major protein bands in the gel of approximate molecular masses 9.5 and 4.2 kDa, which is thus in analogy to the action of pepsin on BLA in its A-state (see above). However, it is seen that the 9.5 kDa protein band almost disappears from the reaction mixture upon a prolonged time of incubation, such as 24 h at 40 °C and that the intensity of the stained protein band of ~4 kDa becomes more abundant. This is also clearly seen when the proteolytic mixtures obtained by incubation for 6 h at 20, 40, or 50 °C are analyzed by SDS−PAGE (Figure 5C). Two main protein bands are seen in the gel with all three samples analyzed, but of different intensities. At 50 °C, the thermolytic cleavage leads mostly to a single, diffuse protein band in the gel (Figure 5C, lane 4).

The identification of the sites of proteolytic cleavage of BLA in its TFE-state by thermolysin was achieved by purification and characterization of the fragments and/or nicked protein species of BLA by micropreparative reverse-phase HPLC on a Vydac C_4 column (Figure 6). The proteolytic sample analyzed was obtained after proteolysis for 6 h at 20 (part A) or 50 °C (part B). Essentially three main peaks of protein material are seen in the HPLC chromatogram, one preceding (Th2) and another following (Th1) that of the intact protein. It is also seen that the material eluted later (Th1) than intact BLA is converted to that eluted earlier (Th2) when the proteolysis is conducted at 50 °C. Analysis by SDS−PAGE of the Th1 sample purified by reverse-phase gave two protein bands of approximate molecular masses 9.5 and 4.2 kDa (not shown). Amino acid analysis after acid hydrolysis (not shown), N-terminal sequencing, and molecular mass analysis (Table 2) allowed us to establish that sample Th1 is a nicked species of BLA, with a single peptide bond fission at Ala40−Ile41 and cross-linked by the four disulfides of the protein (see Figure 1). Moreover, sequence analysis provided evidence of a N-terminal ragged end of Th1, corresponding to partial proteolytic cleavage at the Gln2−Leu3 peptide bond. Thus, the Th1 is a protein sample constituted by two nicked species of BLA, given by disulfide-cross-linked fragments 1−40 and 41−123 and fragments 3−40 and 41−123, in agreement with the two molecular masses given by electrospray mass spectrometry (see Table 2).

Table 2: N-Terminal Sequence and Molecualr Mass of Nicked Species of BLA Obtained by Proteolysis with Thermolysin[a]

	N-Terminal Sequence[b]	
	Th1	Th2
cycle no.	(1−40)(41−123) (3−40)(41−123)	(1−40)(41−80)(81−123) (3−40)(41−80)(81−123)
1	Ile(53)/Glu(39)/Leu(23)	Glu(23)/Leu(102)/Ile(45)
2	Val(67)/Gln(48)/Thr(29)	Asp(19)/Gln(23)/Val(37)/Thr(21)
3	Gln(85)/Leu(61)/Lys(37)	Asp(24)/Gln(28)/Lys(18)/Leu(40)
4	Asn(66)/Thr(35)	Thr(28)/Asp(25)/Asn(24)

Molecular Mass[c]			
Th1		Th2	
14194.5 (14196.8)	13937.1 (13939.6)	14213.8 (14214.8)	13957.5 13957.7)

[a] The nicked species of BLA, named Th1 and Th2, were obtained by proteolysis with thermolysin and purified by reverse-phase HPLC (see text and Figure 6). [b] The results of automatic sequencing are reported as yields (in pmol) of phenylthiohydantoin (PTH) derivative of amino acid recovered on the sequencer at each cycle of Edman degradation. [c] Determined by electrospray mass spectrometry. Calculated values are given in parentheses. Both the Th1 and Th2 nicked species of BLA have a ragged N-terminal end, since thermolysin partially cleaves the peptide bond Gln2−Leu3.

The HPLC-purified Th2 species (see Figure 6B) gave a single diffuse protein band of approximate molecular mass 4 kDa (not shown) and four N-terminal sequences (Table 2). By comparing these sequence data with the known amino sequence of BLA (see Figure 1) and the two molecular masses of the Th2 sample estimated by electrospray mass spectrometry, it was possible to conclude that thermolysin under relatively drastic conditions of proteolysis cleaves at three peptide bonds (Gln2−Leu3, Ala40−Ile41, and Phe80−Leu81). Of note, Th2 contains fragments (1−40, 3−40, 41−80, and 81−123) of very similar molecular masses, explaining the fact that in the SDS−PAGE gel Th2 shows a single, diffuse protein band of ~4 kDa. Additional experiments (not shown) leading to the isolation and further characterization of the individual fragments of both the Th1 and Th2 samples have been conducted. The results obtained confirmed the locations of thermolytic cleavages along the chain of BLA given earlier.

The pattern of protein fragmentation of CAM-BLA by thermolysin in the presence of 50% (v/v) TFE was qualitatively similar to that observed with the intact protein, even if the number of medium size fragments in the SDS−PAGE gels was higher than that observed with the intact protein (not shown). This is in line with the fact that far- and near-UV CD spectra of CAM-BLA in 50% (v/v) TFE are essentially identical to those of the intact protein under the same solvent conditions (not shown).

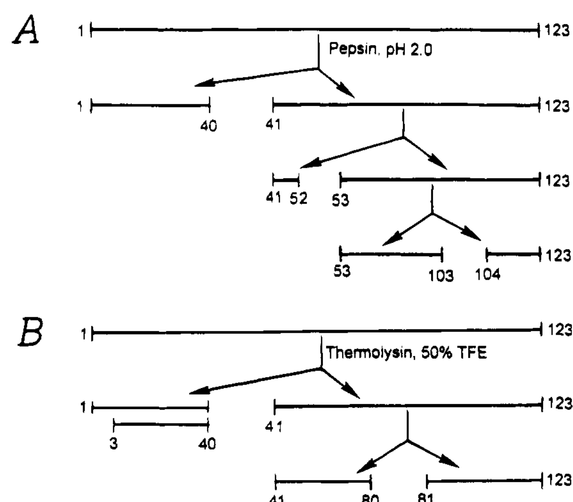

FIGURE 7: Scheme summarizing the time course of the limited proteolysis by pepsin (A) and thermolysin (B) of BLA in its A- or TFE-state.

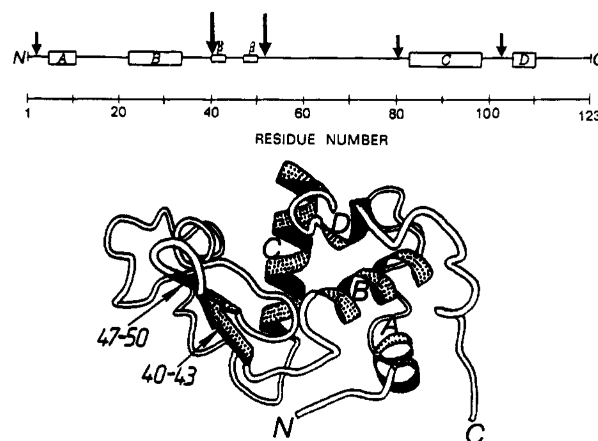

FIGURE 8: Scheme of the secondary structure (top) and three-dimensional structure (bottom) of BLA. The main boxes (top) indicate the helical segments and the minor ones the β-strands of BLA (Acharya *et al.*, 1989, 1991). The sites of fast and slow proteolytic fission of BLA are shown by major and minor arrows, respectively (see text).

DISCUSSION

The results of this study show that the polypeptide chain of BLA in both the A-state and TFE-state can be cleaved by pepsin and thermolysin, respectively, at very few peptide bonds, leading to quite large protein fragments and/or nicked protein species that are rather resistant to further proteolysis, thus allowing their isolation by reverse-phase HPLC. A careful analysis of the data describing the time course of the proteolytic reaction by both pepsin and thermolysin (see Results) allows us to propose that the digestion of BLA is a sequential process, as shown schematically in Figure 7, even if some simultaneous process of proteolysis cannot be ruled out. The initial nicking of BLA in both the A- (pH 2.0) and TFE-states (pH 7.0) occurs at peptide bond Ala40–Ile41. BLA in the A-state is prone to an additional and fast peptic cleavage of the peptide bond Leu52–Phe53, leading to excision from the protein of a 12-residue chain segment and formation of a nicked species constituted by fragments 1–40 and 53–123 cross-linked by the four disulfide bridges of the protein (see Figure 1). The subsequent, but very few cleavages of the chain indicated in the scheme of Figure 7 occur much more slowly.

The ability of BLA in its A- or TFE-state to resist extensive proteolytic degradation seems remarkable, if one considers the broad substrate specificity of both pepsin and thermolysin (Keil, 1982). There are plenty of hydrophobic and bulky amino acid residues in the polypeptide chain of BLA (see Figure 1) that could be attacked by both proteases, if the protein substrate were in a random coil conformation. Considering that proteolysis of a polypeptide chain requires the binding of a chain segment of 6–8 amino acid residues of the substrate at the active site cleft of the proteolytic enzyme (Ottesen, 1967; Berger & Schechter, 1970), this stereospecific binding and adaptation clearly are relatively difficult with BLA even in its partially folded state. Of note, native BLA at pH 7.0 is not attacked by thermolysin, as is often observed with stable globular proteins and as expected from a constrained and rigid fold. Of interest, the lack of contribution of disulfide bonds to the formation of the A-state (Kuwajima, 1977; Ikeguchi & Sugai, 1989) or TFE-state (this study, see Results) of BLA is reflected by the fact that peptic or thermolytic proteolysis of CAM-BLA at pH 2.0 or in 50% (v/v) TFE is similar to that observed with the intact protein.

In previous studies, we have emphasized that surface exposure is not a sufficient characteristic for selective proteolysis, but that chain flexibility is required for a peptide segment to bind at the active site of the protease and to form the idealized transition state of the hydrolytic reaction (Fontana *et al.*, 1986, 1993). For example, it has been shown that proteolytic cleavages along the polypeptide chain of thermolysin occur at exposed loops characterized by high segmental mobility, this last given by the temperature factor (*B*-factor) determined crystallographically (Fontana *et al.*, 1986). Indeed, the overall significant conformational freedom and enhanced flexibility of BLA in either the A- or TFE-state have been demonstrated by comparing amide proton protection (H–D exchange rates) with that of native BLA (Baum *et al.*, 1989; Alexandrescu *et al.*, 1994). Exchange rates of partially folded BLA are much higher than those of native BLA, thus explaining the relative resistance to proteolysis of the native, relatively rigid protein.

The next question is to explain why the chain segment 40–53 of BLA in its A- or TFE-state is the most favored site of proteolytic attack. NMR measurements have indicated that the helical secondary structure of the α-domain of BLA is largely preserved in the A-state of the protein, although without the stereochemical rigidity of the native tertiary structure, whereas the other β-domain (see Figure 8) appears to be missing the structural constraints of the native form (Baum *et al.*, 1989; Chyan *et al.*, 1993). Moreover, the chain segment encompassing the single β-sheet of native BLA (residues 40–43 and 47–50; Acharya *et al.*, 1989, 1991) is highly flexible in the A- and TFE-states of the protein (Alexandrescu *et al.*, 1994). Thus, the fast and selective proteolytic cleavage at chain region 40–53 (see the scheme of Figure 7) is in agreement with the structural and dynamic features of this region deduced by NMR studies. Curiously, far-ultraviolet CD data instead indicate persistence of the β-structure in the molten globule state of BLA [for a discussion of this discrepancy between CD and NMR data, see Alexandrescu *et al.* (1994)].

The analysis of the results of limited proteolysis experiments conducted on globular proteins of known structure reveals that helical segments are never the site of proteolytic attack (Fontana *et al.*, 1993; Polverino de Laureto *et al.*, 1994, 1995). Indeed, in the present case we also do not

observe peptic or thermolytic fragmentation at the level of the helices of native BLA, as seen in Figure 8. BLA in its TFE-state appears to maintain the helical segments of the native species, but the alcohol also induces at least one region of helical structure that does not correspond to a native-like helix in the region 35–41 (Alexandrescu *et al.*, 1994). Thus, thermolysin cleaves at the last residue of the TFE-induced helix, which may be wobbly since Ile41 does not show strong protection from solvent exchange (Alexandrescu *et al.*, 1994).

In order to favor limited proteolysis of BLA in its A-state by pepsin, a relatively low ratio of enzyme to protein substrate had to be employed, such as 1:750 (by weight), along with short reaction times at room temperature. This contrasts with the much more drastic conditions required for conducting the protelysis of BLA by thermolysin in the presence of 50% (v/v) TFE (see Results). We interpret this sluggish reactivity of BLA in its TFE-state as being due to the fact that the content of secondary structure (helix) of the protein substrate in TFE is substantially enhanced with respect to that of both native and A-state of BLA (see Figure 1). The high content of helical secondary structure is expected to hinder proteolysis (see above). However, the slow proteolysis of BLA by thermolysin in aqueous 50% (v/v) TFE is also due to the fact that thermolysin is less active in the presence of organic solvents. The detailed mechanism of thermolysin inhibition is not known, but may involve binding of TFE at its hydrophobic binding site (Matthews, 1988), in analogy to the action of 2-propanol (van den Burg *et al.*, 1989) or the enhancement of protein rigidity in the presence of TFE (Affleck *et al.*, 1992) and, thus, reduction of its catalytic power, since some chain motility is required for catalysis (Welch, 1986). Moreover, the reduced activity of thermolysin can also be explained by considering that proteolytic enzymes in the presence of organic solvents can catalyze the reverse reaction *i.e.*, the synthesis instead of the hydrolysis of the peptide bond (Fruton, 1982).

In summary, this work extends the characterization of a molten globule state of a protein by utilizing a novel approach based on the use of proteolytic enzymes as probes of protein conformation. Present results and conclusions nicely complement and fit those reached on the basis of NMR measurements. This study represents a continuation of our efforts aiming to demonstrate that proteolytic enzymes can be used as reliable probes of protein structure and dynamics, complementing other physicochemical and spectroscopic methods and techniques (Fontana *et al.*, 1986, 1993; Signor *et al.*, 1990; Polverino de Laureto *et al.*, 1994, 1995). Finally, considering that α-lactalbumin is a well characterized model protein currently utilized in a number of laboratories to address fundamental problems in protein structure–stability–dynamics (see the Introduction), the nicked protein species and the fragments described here can be used for additional biophysical studies.

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