
Conformational analysis of HAMLET, the folding variant of human α -lactalbumin associated with apoptosis

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Abstract

A combination of hydrogen/deuterium (H/D) exchange and limited proteolysis experiments coupled to mass spectrometry analysis was used to depict the conformation in solution of HAMLET, the folding variant of human α -lactalbumin, complexed to oleic acid, that induces apoptosis in tumor and immature cells. Although near- and far-UV CD and fluorescence spectroscopy were not able to discriminate between HAMLET and apo- α -lactalbumin, H/D exchange experiments clearly showed that they correspond to two distinct conformational states, with HAMLET incorporating a greater number of deuterium atoms than the apo and holo forms. Complementary proteolysis experiments revealed that HAMLET and apo are both accessible to proteases in the β -domain but showed substantial differences in accessibility to proteases at specific sites. The overall results indicated that the conformational changes associated with the release of Ca^{2+} are not sufficient to induce the HAMLET conformation. Metal depletion might represent the first event to produce a partial unfolding in the β -domain of α -lactalbumin, but some more unfolding is needed to generate the active conformation HAMLET, very likely allowing the protein to bind the C18:1 fatty acid moiety. On the basis of these data, a putative binding site of the oleic acid, which stabilizes the HAMLET conformation, is proposed.

Keywords: HAMLET; α -lactalbumin; conformational analysis; H/D exchange; limited proteolysis

Human α -lactalbumin (human α -LA) is a 14-kD protein whose native structure is made of two lobes: the α -domain (residues 1–34 and 86–123), consisting of four α -helices (A–D) and two short 3_{10} -helices; and the smaller β -domain (residues 35–85), consisting of a triple-stranded antiparallel

β -sheet, a 3_{10} -helix, and a series of loops (Acharya et al. 1991). The holo protein contains a single tightly bound Ca^{2+} and four disulfide bonds, two in the α -domain, one in the β -domain, and one linking the C-helix of the α -domain to the 3_{10} -helix in the β -domain. α -LA has been described to adopt different conformations under different physico-chemical conditions, that is, at low pH (A state) and in the absence of Ca^{2+} (apo). Recently, a folding variant of α -LA that induces apoptosis in tumor and immature cells but not in mature healthy cells was described and named HAMLET (Human Alpha-lactalbumin Made Lethal to Tumor cells; Svensson et al. 1999, 2000). The mechanism of apoptotic induction by HAMLET is still unknown, but it has to be related to the conformation of this folding variant, because

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Abbreviations: CD, circular dichroism; H/D, hydrogen/deuterium; α -LA, α -lactalbumin; MS, mass spectrometry; ESMS, electrospray mass spectrometry; MALDI-MS, Matrix Assisted Laser Desorption Ionization mass spectrometry; LCMS, liquid chromatography mass spectrometry; MS/MS, tandem mass spectrometry.

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neither the apo nor the A-state is able to stimulate apoptosis (Svensson et al. 2000). However, structural details of HAMLET that might account for its specific biological activity are lacking so far.

HAMLET was shown to consist of an α -LA conformer binding a specific fatty acid, C18:1, as a necessary cofactor, and being unable to revert to the holo form under a variety of conditions. It has a native-like secondary structure and larger Stokes radius (Svensson et al. 1999, 2000) than the holo and apo forms, thus showing the typical features of a molten globule-like state. However, near- and far-UV CD, fluorescence, and ANS binding were not able to discriminate between HAMLET and apo- α -LA (Svensson et al. 1999, 2000). The transient nature of these partly folded species and their possible conformational heterogeneity made direct NMR structural studies extremely difficult (Svensson et al. 2000).

In this paper, limited proteolysis and amide hydrogen exchange experiments in combination with mass spectrometry have been used to investigate the surface topology and the conformational flexibility of HAMLET in comparison with the holo and apo forms of human α -LA. Although these approaches provide low-resolution data, they are amenable to the analysis of transient species and partly folded intermediates. Amide protons (H) are exchanged with deuterons (D) with kinetics mainly depending on both solvent

accessibility and the local conformation of the protein backbone (Woodward et al. 1982; Englander and Kallenbach 1983; Li and Woodward 1999). Comparative H/D measurements can then be used to monitor protein structural changes in different experimental conditions (Johnson and Walsh 1994). As deuterons replace protons, the mass of the protein increases, and both the extent and the rate of exchange can then be determined by measuring the increase in protein mass (Katta and Chait 1991; Miranker et al. 1993; Zhang and Smith 1993, 1996; Engen et al. 1999; Halgand et al. 1999; Kim et al. 2002).

Because the distribution of the preferential proteolytic sites is strictly dependent on the protein conformation, limited proteolysis experiments, causing single cleavage events in the native protein structure and originating complementary peptide fragments ("complementary proteolysis"), can be designed to investigate conformational changes in protein structure. This approach was proven to be instrumental in probing protein conformations under different experimental conditions (Bianchi et al. 1999; Orrù et al. 1999; Urbani et al. 1999; Esposito et al. 2000), to monitor quaternary forms interchange (De Lorenzo et al. 1998; Piccoli et al. 2000), to describe protein complexes (Scaloni et al. 1998, 1999; Atkinson et al. 2000), and to characterize partly folded intermediates (Birolo et al. 2002).

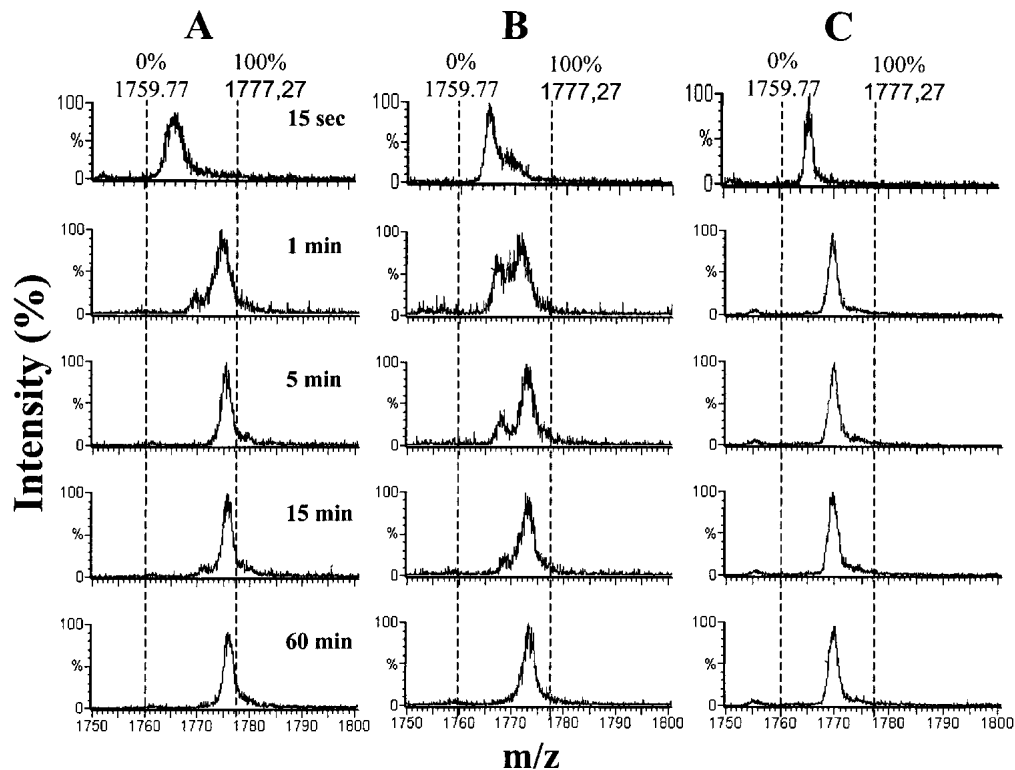


Figure 1. Envelope of isotope peaks at $m/z +8$ in the ESMS spectra of HAMLET (A), apo- α -LA (B), and holo- α -LA (C) at different incubation times in D_2O . The average molecular mass, given by the centroid of each envelope, was used to determine the deuterium incorporation at each incubation time. 0% and 100% indicate the nondeuterated and the fully deuterated m/z values, respectively.

The application of these integrated strategies has clearly shown that HAMLET and apo are two distinct conformers structurally different at the level of the β -domain of the protein.

Results

H/D exchange on intact protein conformations

Comparative H/D exchange experiments were carried out on HAMLET, holo-, and apo- α -LA by sampling protein solutions diluted in the appropriate D₂O buffers at different interval times, and monitoring deuterium incorporation by fast ESMS analysis after cold acid quenching.

Figure 1 shows the envelope of isotope peaks of the +8 ion of HAMLET (Fig. 1A), apo- (Fig. 1B), and holo- α -LA (Fig. 1C) at different incubation times. The single envelope of isotopic peaks exhibited by both HAMLET and holo- α -LA at all incubation times (Fig. 1A,C) indicated an EX2 kinetics of H/D exchange (Bai et al. 1993), and confirmed the conformational homogeneity of the two protein forms, although the widths of the ion peaks, at early incubation times, are suggestive of a higher conformational flexibility in HAMLET.

A quite different picture emerged from the analysis of apo- α -LA. Two envelopes of isotopic peaks could clearly be detected in the mass spectra of apo- α -LA at early incubation times (Fig. 1B), indicating the occurrence of at least two populations of conformers with different exchanging behavior. These populations were in equilibrium and tend to interconvert each other as shown by the presence of a single peak in the late mass spectra when only the heaviest form was observed.

The extent of amide protons exchanged with deuterium in HAMLET, holo-, and apo- α -LA is shown in Figure 2 as a function of time. Data were corrected for back-exchange and reported as the average of triplicate analysis at each time point; differences among measurements never exceeded 2 D. Significant differences in hydrogen exchange were observed among the three forms. The low extent of protons exchanged by holo- α -LA is consistent with a highly structured and homogeneously compact protein, as expected from a native conformation. The hydrogen-exchange profiles of apo- α -LA indicated that one of the two species in equilibrium showed a compact conformation with exchange properties similar to the holo form, whereas the second component should have a flexible structure because it incorporates a higher amount of deuterons. HAMLET exhibited the highest extent of hydrogen deuterium exchange at all the sampled times, suggesting that this species has a conformational flexibility higher than the apo and holo forms. These data support the hypothesis that HAMLET has conformational features similar to a molten globule state (Svensson et al. 1999, 2000).

Flexibility of protein regions

The differences occurring among the conformational states experienced by α -LA were further investigated by proteolytic digestion of the deuterated proteins. HAMLET, holo- α -LA, and apo- α -LA were allowed to exchange for various times and then incubated with pepsin under H/D quench conditions (0°C, pH 2.5). The digested peptide mixtures were directly analyzed by fast LCMS, and data were corrected for back-exchange. Peptic fragments were identified by their unique mass values and MS/MS sequence analysis. Several different peptides corresponding to the same protein regions were observed in the digest owing to the rather broad specificity of pepsin. The nonredundant series of the shortest peptides covering nearly the entire protein sequence was then considered in the analysis (Fig. 3).

Figure 4 shows the percentage of H/D exchange for the selected fragments as a function of the D₂O labeling time of the intact proteins. As expected, all the peptides from holo- α -LA showed an equal or lower degree of deuterium incorporation than those from the apo and HAMLET forms. These results are consistent with previous data on intact proteins, with holo- α -LA showing a more compact and less flexible structure than the two conformers.

When the data on the peptides from apo and HAMLET were compared, a clear difference in the exchange properties of the two domains of α -LA emerged. As shown in Figure 4, panels 1–3, no substantial differences (<10%) in deuterium incorporation were detected in the fragments en-

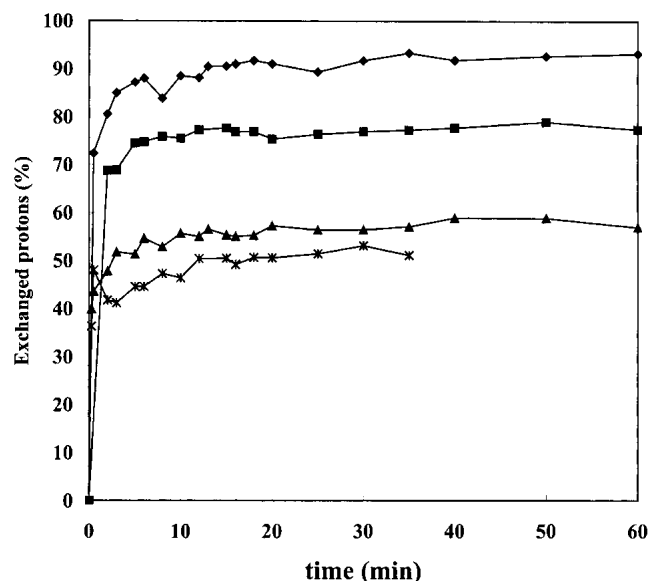


Figure 2. Hydrogen-exchange kinetic profiles of HAMLET (diamonds), apo- α -LA (squares, asterisks), and holo- α -LA (triangles). The amount of exchanged protons at each incubation time was determined from the centroid of the isotopic envelopes in Fig. 1, averaged and corrected with reference samples.

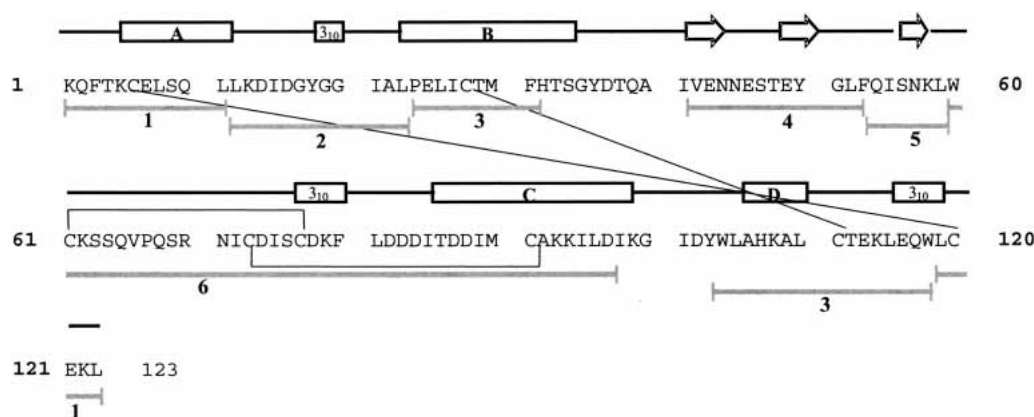


Figure 3. Human α -LA sequence. Secondary structure elements determined by X-ray crystallography (1) are shown above the sequence and the helices A–D are indicated. The pattern of disulfide bonds is represented by lines connecting the corresponding cysteines. Fragments produced by peptic digestion are shown below the sequence and numbered to indicate S–S bridged peptides.

comprising the α -domain, peptides 1, 2, and 3 in Figure 3, corresponding to the sequences 1–11+118–123, 12–23, and 24–31+103–117, respectively. On the contrary, significant differences (>20%) were observed in the fragments 41–52 and 53–59 (Fig. 4, panels 4 and 5), corresponding to the β -strands constituting the core of the β -domain of α -LA (peptides 4 and 5 in Fig. 3). In this region, HAMLET incorporated a higher percentage of deuterium with faster kinetics as compared with apo, thus indicating that conformational differences exist in the β -domain of the two conformers. A difference in the HD exchange properties between apo and HAMLET was also observed in the peptide 60–96 (Fig. 4, panel 6). However, this large fragment encompasses residues from both the β - (60–85) and the α -domains (85–96, corresponding to the C-helix), making it difficult to determine which region is responsible for the different behavior observed.

Limited proteolysis experiments

The surface topology of HAMLET was probed by limited proteolysis experiments using trypsin, chymotrypsin, V8 and AspN endoproteases, subtilisin, and endoprotease K as conformational probes. Protein samples were incubated with each protease using an appropriate enzyme-to-substrate ratio, selected to achieve single proteolytic events on the intact protein. In all cases, under the experimental conditions used, the protein remained largely undigested, with only a limited number of fragments released from the protein molecule, showing that this conformation is accessible to proteolysis at a few very specific sites. The extent of the enzymatic hydrolysis was monitored on a time-course basis by sampling the incubation mixture at different interval times followed by HPLC fractionation. The fragments generated from the protein were identified by electrospray mass spectrometry, leading to the assignment of cleavage sites.

Figure 5, A and B, shows the HPLC chromatograms of the aliquots withdrawn after 15 and 30 min of endoprotease V8 digestion of HAMLET. The ESMS analysis of peak 2 showed a mass value of $14,088 \pm 1.2$ D, 18 D higher than the mass value of α -LA, thus accounting for the introduction of a water molecule. Peak 2 was then identified as a nicked form of the protein in which the V8 protease had cleaved a single peptide bond but the protein was still maintained intact by the four S–S bridges. The preferential cleavage site was assessed by reduction and alkylation of the intrachain disulfide bridges, followed by LC-ESMS analysis of the fragments. Mass spectral analysis showed the presence of two peptide pairs, 1–46/47–123 and 1–49/50–123, indicating the occurrence of two independent cleavages at Glu 46 and Glu 49.

Similar results were obtained using the specific proteolytic enzymes trypsin and endoprotease Asp-N, indicating preferential cleavage sites at Arg 70 and Asp 37, respectively.

Proteolytic experiments were also carried out using broader specificity proteases, leading to the identification of Tyr 50, Gln 39, and Phe 53 as preferential proteolytic sites. As an example, the ESMS analysis of the HPLC fractions following incubation of HAMLET with proteinase K identified the peptide pair 1–39/54–123 and the small fragment 40–53. The absence of either the fragments 1–39/40–123 or the peptides 1–53/54–123 indicated that cleavages had almost simultaneously occurred at Gln 39 and Phe 53.

Limited proteolysis experiments were likewise performed on apo- and holo- α -LA using the same panel of proteolytic enzymes. The holo protein was completely resistant to proteolysis under conditions in which both apo and HAMLET were sensitive to proteolytic cleavages. Because the compact form of apo exhibits an H/D behavior very similar to the holo- α -LA, we suggest that only the more flexible state of apo- α -LA could be probed by limited proteolysis

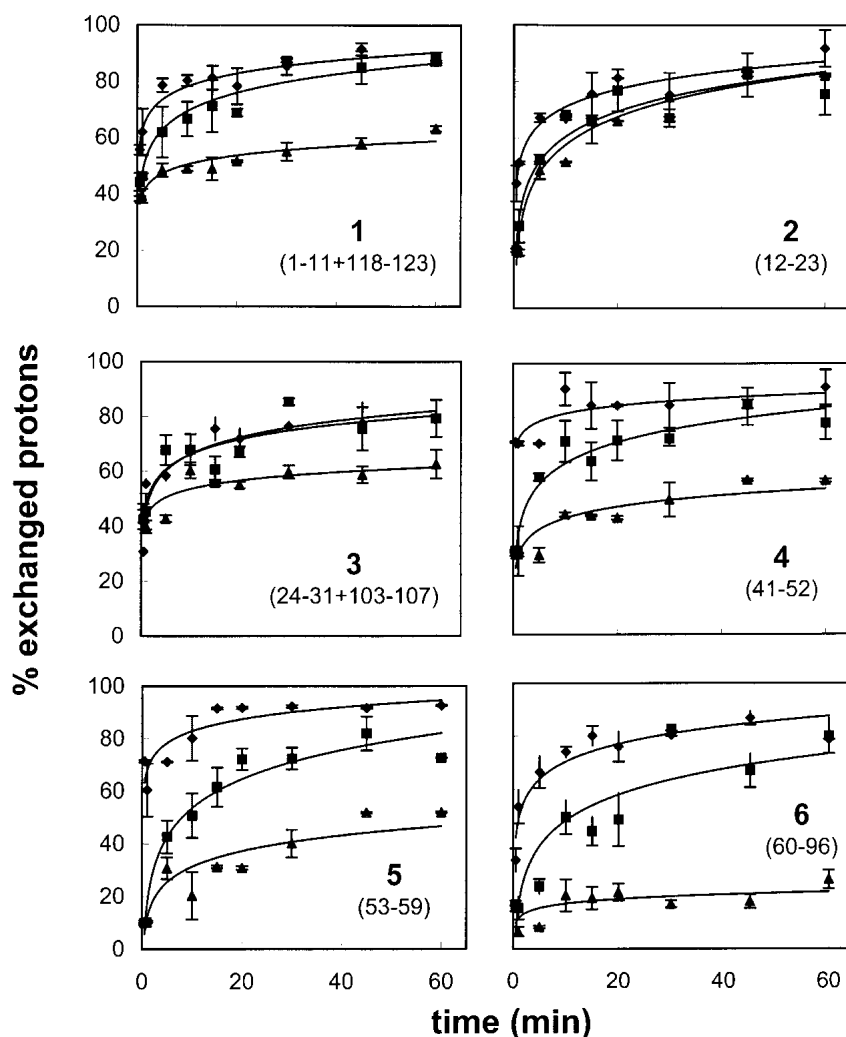


Figure 4. Hydrogen-exchange kinetic profiles of peptides generated by peptic digestion of deuterated HAMLET (diamonds), apo- α -LA (squares), and holo- α -LA (triangles). The fragments 1-11+118-123 (*J*) and 24-31+103-117 (*J*) consist of peptides 1-11 and 118-123 linked by the disulfide bridge between Cys 6-Cys 120, and 24-31 and 103-117 linked by the S-S bridge between Cys 28-Cys 111, respectively. The number of protons exchanged at each time was determined from the centroid of the isotopic envelopes, averaged, and corrected with reference samples. Lines through data were drawn simply to guide the eye.

experiments, being the more compact form very likely inaccessible to proteases.

The overall results of the limited proteolysis experiments obtained on HAMLET and apo are summarized in Figure 6, where the optimal E : S ratio selected for each experiment is also reported. An identical distribution of preferential cleavage sites was observed for the two Ca^{2+} -depleted conformations with all the recognized residues located in the β -domain, in agreement with the H/D data previously described.

However, the existence of small conformational differences between the two protein species could be inferred by the different E : S ratios needed to properly control the activity of proteases in the various experiments (Fig. 6). These differences were further investigated by performing proteolytic experiments in parallel on HAMLET and apo- α -LA

using the same E : S ratio. As an example, Figure 6 shows the HPLC profiles after 60 min of incubation of HAMLET (Fig. 6C) and apo- α -LA (Fig. 6D) with proteinase K at the same E : S ratio (1 : 500). Under these conditions, apo- α -LA was rapidly cleaved at Gln 39 and Phe 53, whereas HAMLET was essentially undigested. Similar results were observed with trypsin and subtilisin, clearly showing that Arg 70 and Phe 53 are more accessible in apo than in HAMLET. Digestion with endoprotease V8 showed an almost equal accessibility of the two proteins at Glu 46 and Glu 49, whereas the experiments with chymotrypsin and endoprotease AspN showed an opposite behavior of the two conformers. Digestions at Tyr 50 and Asp 37, in fact, occurred faster in HAMLET than in apo- α -LA, suggesting a higher accessibility of these residues in the apoptotic con-

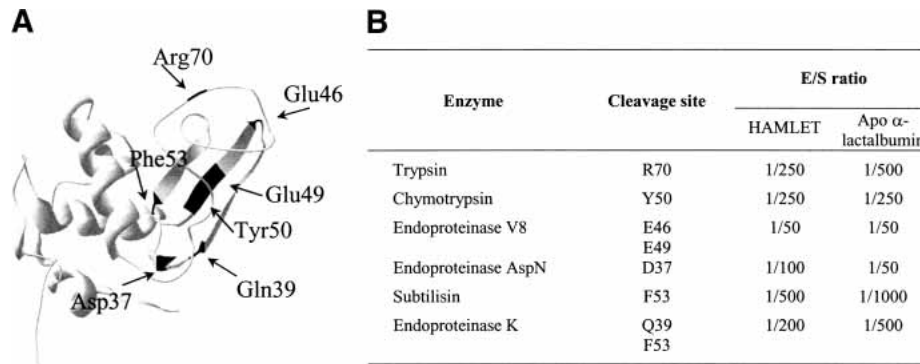


Figure 5. (A) Preferential cleavage sites observed in HAMLET and apo- α -LA mapped on the three-dimensional structure of holo- α -LA (1A4V). (B) Summary of limited proteolysis results, with the indication of the E : S ratios used to control protease action in each experiment.

formation. As a whole, these data underlined the occurrence of different accessibility/flexibility in the two conformers depending on the specific proteolytic site, again suggesting a slightly different organization of the β -domain in the two Ca^{2+} -depleted conformations.

Discussion

Modern views in structural biology (Mann et al. 2001) have already recognized that protein flexibility is a key feature not only in contributing to the catalytic activity but also in

conferring special biological functions. Nonnative states experienced by proteins *in vivo* are well known to play a major role in protein association and aggregation, leading to the so-called conformational diseases (Dobson and Karplus 1999; Chiti et al. 2000). HAMLET is a striking example of a nonnative conformation endowed with an acquired biological activity. This conformer that is stabilized by a fatty acid cofactor is able to induce apoptosis in tumor cells, thus possibly conferring exquisite antitumoral properties to human milk, whereas this function cannot be exerted by both holo- and apo- α -lactalbumin (Svensson et al. 1999, 2000).

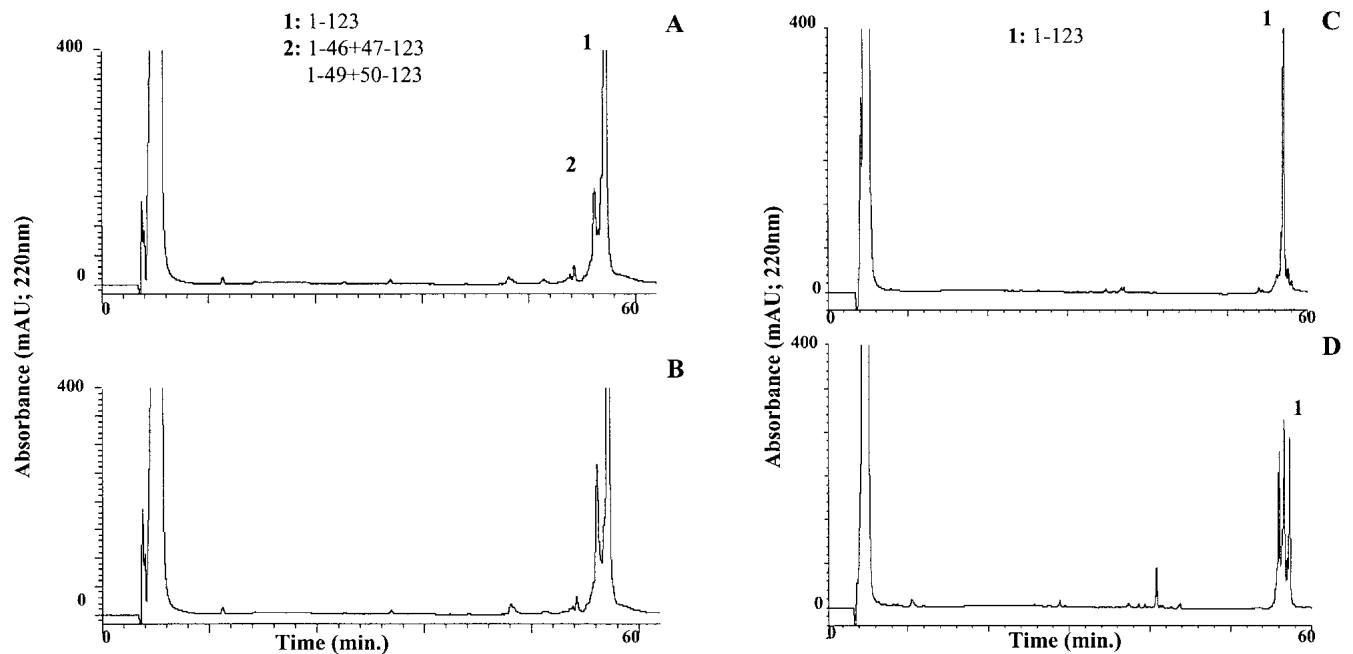


Figure 6. (A,B) Time-course analysis of endoprotease V8 digestion of HAMLET under controlled conditions using an E : S ratio of 1 : 50 (w/w). HPLC profiles of the aliquots withdrawn from the incubation mixture at 15 min (A) and 30 min (B). Individual fractions were collected and identified by ESMS and LCMS analysis after reduction and alkylation; the corresponding peptides are reported in the inset. (C,D) Comparison of the HPLC profiles of the aliquots withdrawn after 60 min of incubation with proteinase K of HAMLET (C) and apo- α -LA (D), using an E : S ratio of 1 : 500 (w/w).

The results presented here identified structural features that may account for the peculiar biological activity of HAMLET and confirmed at the molecular level that this conformer is indeed different from apo- α -LA. The H/D experiments clearly indicated that under the conditions used, apo- α -LA exists as a mixture of at least two populations of conformers with different exchanging properties, a very compact species and a more flexible component. HAMLET thus differed from any of the apo conformers, because it incorporated a sensibly higher number of deuterium atoms even when compared with the more flexible species of apo- α -LA.

Location of the conformational differences between HAMLET and the flexible form of apo- α -LA was obtained by peptic hydrolysis of the deuterated proteins. Both conformers exhibited a higher accessibility and/or flexibility in the β -domain as compared with the holo protein, whereas no substantial differences could be observed in the α -domain. Major differences between HAMLET and apo were detected in the peptides 41–52 and 53–59, encompassing the three antiparallel β -strands, where HAMLET exchanged a rather higher number of amide protons than apo and exhibited a higher kinetics of exchange.

These results indicated that the conformational changes associated with the release of Ca^{2+} are not sufficient to allow fatty acid binding and induce the HAMLET conformation. H/D exchange data clearly demonstrated that some more unfolding in the β -strand region of the β -domain occurred in HAMLET as compared with apo- α -LA. This further unfolding was very likely needed to accommodate the C18:1 fatty acid moiety, the necessary cofactor that stabilizes the active conformation. Metal depletion might then represent the first event to produce a partial and localized unfolding of the polypeptide chain in the β -domain that is needed to acquire enough conformational flexibility to bind oleic acid and to gain the new biological function. This finding is consistent with the observation that the apo form of α -LA cannot be converted to HAMLET by simply mixing the protein with oleic acid, but a “conditioning” chromatographic step is needed to optimize the apoptosis-inducing function (Svensson et al. 2000).

Further structural details on the HAMLET conformation were obtained by limited proteolysis experiments using a panel of different proteases. Under the experimental condition used, only a limited number of fragments were released from the protein, showing that HAMLET is sensitive to proteolysis at few very specific sites. These findings confirm that HAMLET has a flexible but stable and structured protein conformation, as previously assessed by CD spectroscopic investigation (Svensson et al. 1999, 2000). The apparent contradiction between the proteolytic resistance and the high extent of H/D exchange observed in HAMLET might be explained in terms of protein dynamics rather than static structures. We suggest that HAMLET experiences a

wide protein breathing on a time scale consistent with H/D exchange reaction but much faster than that required for the enzyme–substrate recognition necessary for the proteolytic event. Indeed, H/D exchange experiments were even able to detect differences between proteins with superimposable X-ray structures (Birolo et al. 2002).

These results were then compared with those obtained on apo- α -LA used as reference. All the preferential proteolytic sites detected in both apo and HAMLET gathered within the β -domain, in agreement with H/D exchange data. Moreover, an identical distribution of proteolytic cleavages was observed for the two Ca^{2+} -depleted forms. In both protein species, in fact, enzymatic cleavages occurred within the three antiparallel β -strands (residues 41–43, 48–50, and 55–56) and the connecting loops constituting the β -sheet structure, with the exception of Arg 70 located in the long loop connecting the β -sheet to the 3_{10} -helix.

However, a qualitative kinetic evaluation of these experiments, essentially based on the different E : S ratios needed to control protease activity, underlined a different behavior of the two conformers at specific sites. In particular, proteolytic cleavages at Tyr 50 and Asp 37 occurred much faster (i.e., at lower E : S ratios) in HAMLET than in apo. In native α -LA, Tyr 50 is located within the central β -strand and is hydrogen-bonded to Ile 55 of strand 3. This interaction, together with the hydrogen bond between Glu 49 and Val 42 of strand 1, is essential for the optimal pairing of the three strands within the β -sheet. The accessibility of Tyr 50 to the proteases might then be related to the partial unfolding of the β -domain following calcium release. The higher proteolytic susceptibility of this residue in HAMLET suggested a further distortion in the structure and packing of the central β -strand. This event might be essential for the fatty acid binding, with the aliphatic chain of oleic acid: (1) intercalating within the β -sheet; (2) displacing the central β -strand; and (3) exposing Tyr 50 to protease action. This hypothesis was further supported by the higher accessibility to proteases exhibited by Asp 37 in HAMLET as compared with apo. The side chain of this residue makes contacts with the peptide bonds of Thr 38 and Gln 39. In the HAMLET complex, the carboxyl moiety of the fatty acid might replace the β -COOH group of Asp 37, thus exposing this residue to proteolytic enzymes, and possibly making stabilizing interactions with the same residues or with either the ϵ - NH_2 or the α - NH_2 of Lys 1.

The present study shows how low-resolution approaches might be instrumental in providing subtle structural details on partly folded conformations, such as HAMLET. These procedures were able to detect structural differences that suggest a distinct conformation for HAMLET, possibly following the oleic acid binding. On a more general ground, structural genomics projects aimed at investigating on conformational changes associated with different biological functions of the same polypeptide chain can greatly benefit

of the limited proteolysis, H/D exchange, and mass spectrometry combined strategies.

Materials and methods

HAMLET and the holo and apo forms of human α -LA were prepared as previously described (Svensson et al. 2000), and all the resulting preparations were homogeneously monomeric (data not shown). HAMLET apoptotic-inducing activity was tested before structural analysis as previously described (Svensson et al. 2000). Trypsin TPCK-treated, chymotrypsin, subtilisin, and endoprotease K were purchased from Sigma, endoprotease V8 and AspN from Boehringer Mannheim, and RP-HPLC columns C4 and C18 (250 \times 2.1 mm, 300 Å pore size) from Phenomenex. All other reagents and solvents were HPLC-grade from Carlo Erba and Baker Mallinckroft.

H/D exchange

The H/D exchange reaction was conducted as follows: The protein sample (1.2 nmole/ μ L) was allowed to equilibrate for 15 min at 25°C in 10 mM ammonium acetate (pH 7.0). Deuterium exchange was initiated at 25°C by 10-fold dilution with 10 mM ammonium acetate in D₂O (pD 7.0). At various exchange times (30 sec to 1 h), ~3 nmole of protein was removed from the labeling solution, rapidly diluted with ice-cold buffer at 1:5 to obtain 20% CH₃CN, 0.1% TFA (pH 2.0), and analyzed after 1 min of back-exchange at 0°C by ESMS on a ZQ single quadrupole instrument (Micromass; 2.7 capillary voltage, 50 cone voltage, 80°C source and desolvation temperature). The deuterium content was deduced from the increase in molecular mass; spectra were acquired and processed by centroiding an isotopic distribution using the Mass-Lynx program. Two control samples, that is, undeuterated α -LA (m_0) and fully deuterated α -LA (m_{100}) were analyzed under the same conditions. Equation 1 was used to correct for back-exchange:

$$D = (m_t - m_0)/(m_{100} - m_0) \times 100 \quad (1)$$

where D is the percentage of deuterium content, m_t , m_0 , and m_{100} are the average molecular mass of the same protein species in the partially, undeuterated, and fully deuterated forms. Fully deuterated α -LA was obtained by incubating 10 nmole dissolved in 200 μ L of D₂O for 12 h at 50°C, lyophilizing the sample, and incubating it for a further 12 h at 50°C in D₂O to a final concentration of 0.12 mM.

Pepsin digestion of protein deuterated samples (see above) was performed by fourfold dilution of each sample (6 nmole) with a 0.1% TFA (pH 2.5) containing 169 μ g of pepsin and incubating for 5 min at 0°C. Peptides were analyzed by LCMS on the ZQ instrument coupled to a 2690 Alliance HPLC, Waters using a 30 \times 0.46 mm i.d. reverse-phase perfusion column (POROS 10 R2; Applied Biosystems). Peptides were eluted at a flow rate of 0.5 mL/min with a 10%–60% CH₃CN gradient in 0.1% TFA in 8 min. Mass spectrometry data were obtained, elaborated, and corrected for back-exchange as previously described. Then 3 nmole of non-deuterated holo- α -LA was digested with pepsin as above and fractionated using a 250 \times 2.1-mm, 300 Å Phenomenex Jupiter C18 column on an HP 1100 HPLC (Agilent Technologies) coupled to an LCQ ion trap (Finnigan Corp.) to confirm peptide assignments by MSMS data.

Limited proteolysis experiments

Enzymatic hydrolysis were performed at 25°C by incubating the sample in 50 mM Tris-HCl (pH 7.5; in the presence of 10 mM EDTA for HAMLET and apo- α -LA) with enzyme-to-substrate ratios ranging from 1 : 1000 to 1 : 50 (w/w). The extent of proteolysis was monitored on a time-course basis by sampling the reaction mixture at different time intervals from 15 to 60 min. Digested protein samples were acidified to pH 2.5 by adding TFA, and proteolytic fragments were separated by reverse-phase HPLC on a Phenomenex Jupiter C18 column (250 \times 2.1 mm, 300 Å pore size) with a linear gradient 15%–65% acetonitrile in 0.1% TFA over 55 min, at a flow rate of 200 μ L/min. Elution was monitored at 220 nm and 280 nm. Individual fractions were collected and analyzed by ESMS using an API-100 single quadrupole instrument (Applied Biosystems). When necessary, identification of disulfide-bridged fragments was carried out by reduction with dithiothreitol and alkylation with iodoacetamide (Nitti et al. 1995) followed by RP-HPLC separation and ESMS analysis. Data were acquired and processed using the Biomultiviewer (Applied Biosystems) software.

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