Articles

Structure of the Membrane-Bound Form of the Pore-Forming Domain of Colicin A: A Partial Proteolysis and Mass Spectrometry Study

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ABSTRACT: The ion-channel-forming thermolytic fragment (thA) of colicin A binds to negatively charged vesicles and provides an example of the insertion of a soluble protein into a lipid bilayer. The soluble structure is known and consists of a 10-helix bundle containing a hydrophobic helical hairpin. In this study, partial proteolysis and mass spectrometry were used to determine the accessible sites to proteolytic attack by trypsin and α-chymotrypsin in the thA fragment in its membrane-bound state. Electrospray mass spectrometry was quite an efficient method for the identification of the cleavage products, even with partially purified peptide mixtures and with only few controls by N-terminal sequencing. This work confirms that a major part of the peptide chain lies at the membrane surface and that even the hydrophobic hairpin is not protected by the lipid bilayer from proteolytic degradation. In the absence of a membrane potential, the hydrophobic hairpin in the colicin A membrane-bound form seems not fixed in a transmembrane orientation.

The recently solved crystal structure of the channel-forming thermolytic fragment of colicin A (thA) (Parker et al., 1989, 1992) has revealed for the first time at high resolution the structure of a protein which inserts spontaneously into lipid bilayers (Pattus et al., 1983; Massotte et al., 1989; Frenette et al., 1989). This structure can be described as a bundle of 10 α-helices which are arranged in three layers (Figure 1A). The middle layer consists of a hydrophobic helical hairpin completely buried within amphipathic helices. The way in which this polypeptide of 204 residues, which is highly water soluble, alters its conformation to become an integral membrane protein and eventually an ion channel is a fascinating problem in protein folding. Channel formation by colicins *in vitro* is a two-step process: (i) spontaneous binding of the whole colicin or its thermolytic fragment to a negatively charged bilayer catalyzed *in vitro* by acidic pH and (ii) ion channel formation induced by membrane potential. We have recently shown that colicin A undergoes a native-to-molten globule transition at acidic pH (van der Goot et al., 1991; Gonzàlez-Mañas et al., 1992) and that the kinetics of insertion of the protein into negatively charged lipid vesicles correlates well with the appearance of this acidic molten globular state. This state is therefore a membrane insertion intermediate. Moreover, after insertion, the protein remains in a molten globule-like conformation (Muga et al., 1993).

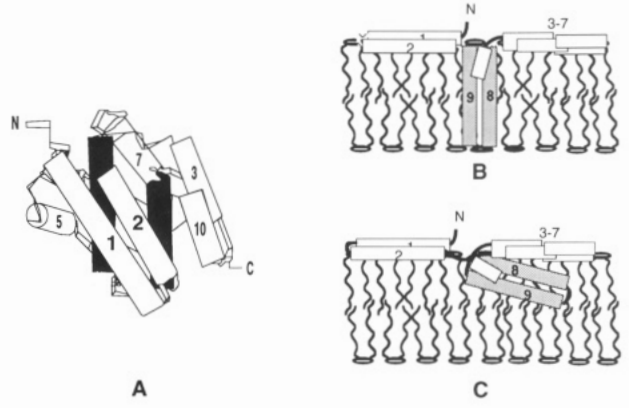

FIGURE 1: X-ray structure of the soluble form and the two proposed models of the membrane-bound form of the pore-forming domain of colicin A. (A) X-ray structure [taken from Parker et al. (1989, 1992)]. (B) The umbrella state (Parker et al., 1990, 1992). (C) The penknife model (Lakey et al., 1993).

What structural change ensures that the colicin is anchored in the membrane? The protein is strongly bound to the lipids and does not exchange between vesicles (Gonzàlez-Mañas et al., 1993). It therefore conforms to criteria expected of integral membrane proteins. The presence of a very hydrophobic hairpin in colicin is a tempting answer to the anchoring requirement, as the free energy gain on insertion could catalyze the unfolding of the molten globule structure. A model for this process has been proposed by Parker et al. (1990) and was based upon evidence from biochemistry (Massotte et al., 1989; Slatin et al., 1986), spectroscopy (Lakey et al., 1991b), and thermodynamic considerations (Engelman & Steitz, 1981). In this model the three layers of the crystal structure have been rearranged so that the complete hydrophobic hairpin formed by helices 8 and 9 spans the lipid bilayer perpendic-

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ularly to the membrane plane, leaving helices 1 and 2 on the opposite side to and a significant distance away from helices 3–7 (Figure 1B).

However, recent fluorescent energy-transfer experiments (Lakey et al., 1993) and engineering of disulfide bonds between the hydrophobic hairpin and neighboring helices in the soluble structure (Duché et al., 1993) have cast doubt about the transmembrane orientation of the hydrophobic hairpin. Both studies suggest strongly that helices 8 and 9 are in a similar packing arrangement in the soluble and the membrane-bound structures. This points to an unfolding of the molecule into a penknife rather than an umbrella model in which helices 3–10 remain in a similar but looser conformation compared to that in solution (Figure 1C). The removal of helices 1 and 2 exposes the hydrophobic faces of helices 8 and 9 which bind to but do not penetrate the membrane phase. Helices 1 and 2 lie with their hydrophobic faces exposed to the membrane just like in the umbrella model (Lakey et al., 1993).

In the present study, an independent approach was taken to probe the accessibility of peptide bonds at the surface of the membrane by combining partial proteolysis, mass spectrometry, and peptide sequencing. This work confirms that a major part of the peptide chain lies at the surface of the bilayer and suggests that the hydrophobic hairpin can be cleaved by α-chymotrypsin from the outside of the membrane vesicle.

MATERIALS AND METHODS

Cell Growth and Purification. Colicin A was purified from *Citrobacter freundii* strain CA31 and converted into its thermolytic fragment as described previously (Cavard & Lazdunsski, 1979; Tucker et al., 1986). The C-terminal peptide was purified by filtration on a Sephadex G50 column, dialyzed against water, and lyophilized. The lyophilized peptide was dissolved in the desired buffer, and the solution was spun in an Eppendorf centrifuge for 15 min to remove suspended particles and nonsolubilized material. The thermolytic fragment concentration was determined from the absorbance at 280 nm using the molar absorption coefficient ($\epsilon_{280} = 2.43 \times 10^4\ M^{-1}\ cm^{-1}$) determined previously (Massotte et al., 1989).

Preparation of Lipid Vesicles and DMPG/Peptide Complexes. Dimyristoylphosphatidylglycerol (DMPG) was purchased from Avanti Polar Lipids Inc., gave a single spot on thin-layer chromatograms using $CHCl_3/MeOH/H_2O$ (65/25/4 v/v/v) as migration solvent, and was used without further purification. Twenty milligrams of phospholipid was sonicated above the transition temperature with a bath sonicator (Bandelin, Sonorex TIX 30) in 1 mL of HEPES (50 mM), adjusted to pH 7.2 with NaOH. The lipid–peptide samples were prepared by mixing a peptide solution (2 mg/mL final concentration) and the appropriate volume of DMPG vesicles to obtain an R_i value of 100 (number of lipid molecules per molecule of peptide). The mixtures were then incubated for 10 min at 37 °C.

Proteolytic Digestion. Unless otherwise stated, the lipid/protein complex at R_i = 100 was digested with 1% (w/w) either TPCK-treated trypsin from bovine pancreas (Serva) or TLCK-treated α-chymotrypsin from bovine pancreas (Sigma). Unless otherwise stated, each sample was incubated for 10 min at 37 °C and proteolysis was stopped by acidification with HCl (1 M) to pH 4.0.

Delipidation. The digested complex was precipitated by addition of 5 mM $CaCl_2$ and spun down at 16 000 rpm for 30 min in an SS34 rotor. The pellet was first dried under nitrogen and solubilized in at most 1 mL of chloroform/methanol (1/1 v/v) containing 5% 0.1 M HCl. Lipids and peptides were then separated by gel filtration chromatography on an LH60 column (50 × 1 cm) for use in organic solvents according to the method of Boyot et al. (1989). The column was monitored by the optical density at 280 nm. Fractions of 500 μL were collected and immediately dried. Proteins in the fractions were separated on 8 M urea SDS–PAGE (15% acrylamide, 3.75% bisacrylamide, stacking gel 5%) and stained by silver staining. Lipids were analyzed by TLC on DC-60 plates with chloroform/methanol/water (65/25/4) as migration solvent and stained first with iodine and then with molybdate blue (Diettmer & Lester, 1964). Lipids were also estimated by phosphorus assay according to the method of Bartlett (Chen et al., 1956).

Electrospray Mass Spectrometry. Electrospray mass spectrometry (ES-MS) was performed on a VG BIO-Q (Fisons) quadrupole mass spectrometer with a mass range of 4000. The mass spectrometer was scanned from m/z = 500 to m/z = 1500 in 10 s. Data were acquired by operating the data system as a multichannel analyzer, and several scans were summed to obtain the final spectrum. The instrument was calibrated with horse heart myoglobin (5 pmol/μL, 16 950.5 Da) from Sigma.

The electrospray ion source was operated as described (van Dorsselaer et al., 1990). Samples (10 μL) were injected into the electrospray source at a flow rate of 5 μL/min as a solution (typically 20/pmol/μL) in water/methanol (50/50 v/v) containing 1% acetic acid.

Determination of Proteolytic Cleavage Sites. Proteolytic fragments were screened with respect to molecular weight values (average mass) obtained by ES-MS against the expected molecular weights according to the sequence of the thA fragment (204 amino acids) with the help of special prediction software (Biopolymer Tools, VG Biotech, Fisons) provided on the mass spectrometer. This program searches for a contiguous stretch of thA amino acid sequence the molecular weight of which matches best the experimental molecular weight. The unique fragments were then validated by checking whether they correspond to tryptic or α-chymotryptic specific cleavage sites.

The nearest candidates screened from the molecular weight (M_w) value had generally at both N-terminal and C-terminal sides the expected cleavage sites according to the enzyme's specificity. If any fragment could not be identified in this manner or if more than two fragments happened to be possible for one molecular weight value, we specified these fragments by sequencing some fractions containing them on an Applied Biosystems protein sequencer (Model 473A).

Independently, in order to compare the performance of the ES-MS strategy to more conventional methods, the cleaved thA/DMPG mixture was solubilized in SDS and the peptides were separated by 8 M urea SDS–PAGE electrophoresis (stacking gel 5%, sepearation gel 15% acrylamide, 3.75% bisacrylamide). The major tryptic and α-chymotryptic fragments were then N-terminally sequenced after electrotransfer on polypropylene PP20 (Schleicher-Schull) as described by Matsudaira (1987).

RESULTS

The general scheme for the determination of proteolytic fragments from thCa31 is shown in Figure 2. The pore-forming fragment was first reconstituted with DMPG vesicles (Massotte et al., 1989) and subjected to protease treatment. The peptides were then delipidated and analyzed by mass

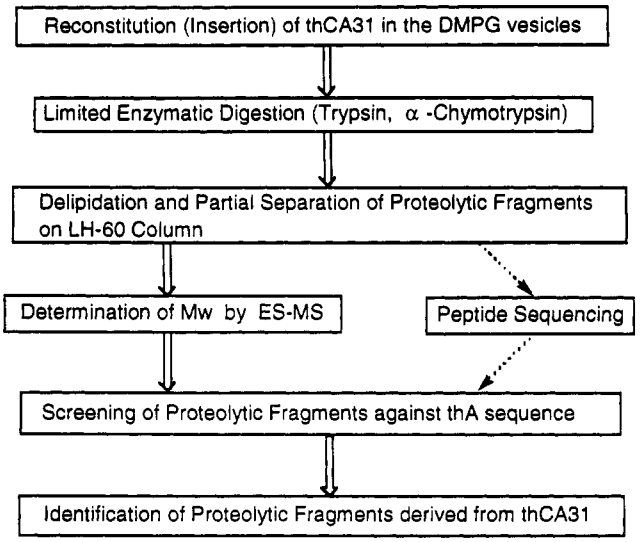


FIGURE 2: General strategy for the determination of proteolytic cleavage sites on the pore-forming domain of colicin A bound to DMPG vesicles.

spectrometry and peptide sequencing whenever necessary. Several controls of the different steps had to be done before the final analysis.

Kinetics of Digestion by Trypsin and α-Chymotrypsin with and without Lipids. Comparison of the kinetics and the extent of digestion by trypsin of the peptide in solution or bound to DMPG vesicles shows a profound difference in sensitivity of the two forms of the peptide. At a ratio of 1/100 trypsin/peptide, cleavage products of the soluble form appear only after 4 h of incubation, whereas the membrane-bound form is already partially cleaved after 2 min of incubation (Figure 3, top and middle). With α-chymotrypsin, the soluble form is almost completely resistant to proteolytic cleavage even at a ratio of 10/100 (enzyme/protein, data not shown), but the membrane-bound form is highly sensitive (Figure 3, bottom). After prolonged incubation, both enzymes degrade the membrane-bound fragment down to very small fragments. This is unexpected for trypsin, since the fragment contains a long hydrophobic stretch devoid of putative cleavage sites for trypsin. One would have expected the formation of a 7 kDa fragmenmt insensitive to further degradation. It has been shown in other systems that TPCK-treated trypsin could have intrinsic chymotrypsin-like activity different from a contamination by chymotrypsin (Inagami & Matsuda, 1964; Plapp et al., 1967). This nevertheless implies that after prolonged incubation trypsin has access to the hydrophobic hairpin.

In order to claim that the observed cleavage sites are indeed accessible to the protease in the intact membrane-bound form of thA, it was necessary to avoid as much as possible reorganization of the structure after several cleavages had occurred. In other words, it is necessary to stop the proteolytic reaction as soon as the first cleavage events are visible (t = 5–10 min, Figure 3, middle and bottom). The liposome structure is preserved upon acidification to stop the reaction. One could expect that some hydrophilic peptides generated by proteolytic cleavage may be released during digestion or are loosely associated to the membrane. We checked that all the peptides remain associated to the vesicles after gel filtration, sedimentation in a sucrose gradient, or centrifugation (data not shown). They are all soluble in acidic chloroform/methanol mixtures. Therefore, no topographical information could be obtained from these rough treatments. This suggests only that a large part of the polypeptide is in direct and strong interaction with the lipids.

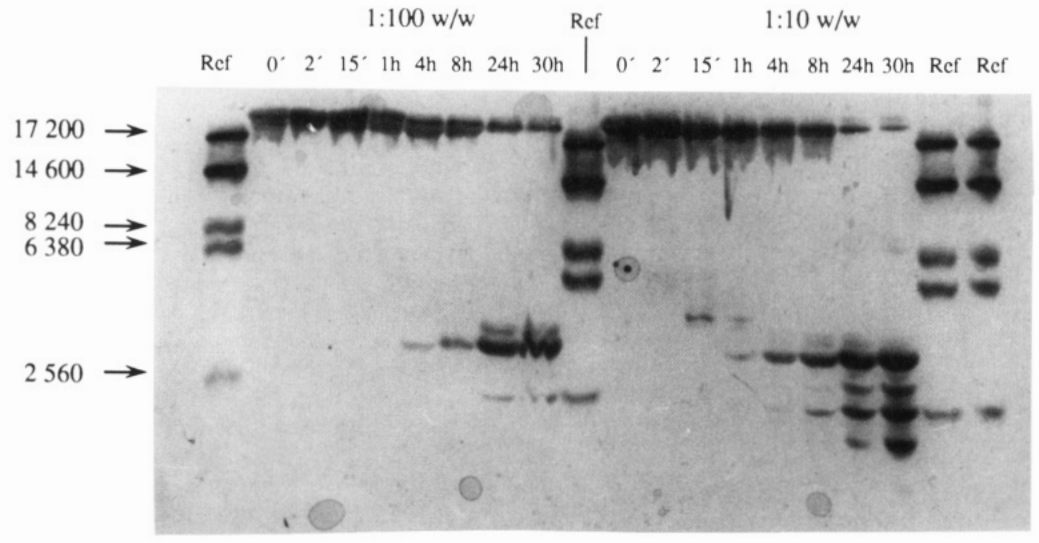
Molecular Weight of the Pore-Foming Domain of Colicin A (th CA31) before and after Delipidation. Initially, we measured the molecular weight (M_w) of the intact thA fragment by ES-MS (Figure 4), shown to be M_w = 21 790.08 ± 1.96, in good agreement with the value deduced from the sequence (Table I). Furthermore, the minor component of the thA lacking two amino acids at the C-terminus was found in the spectrum, in agreement with previous studies showing that part of the fragment was lacking the C-terminal histidine (Massotte, 1991). Secondly, we checked whether any methylation occurred during the delipidation step on the LH60 column because methanol and HCl were used in the eluent to increase the solubility of the peptide. When the solvent was removed from the fractions immediately after elution, methylation was kept to a minimum and could be neglected. This allows the determination of a correct M_w value for the intact fragment after delipidation (Table I) and suggests that ES-MS will perform similarly with the cleavage products.

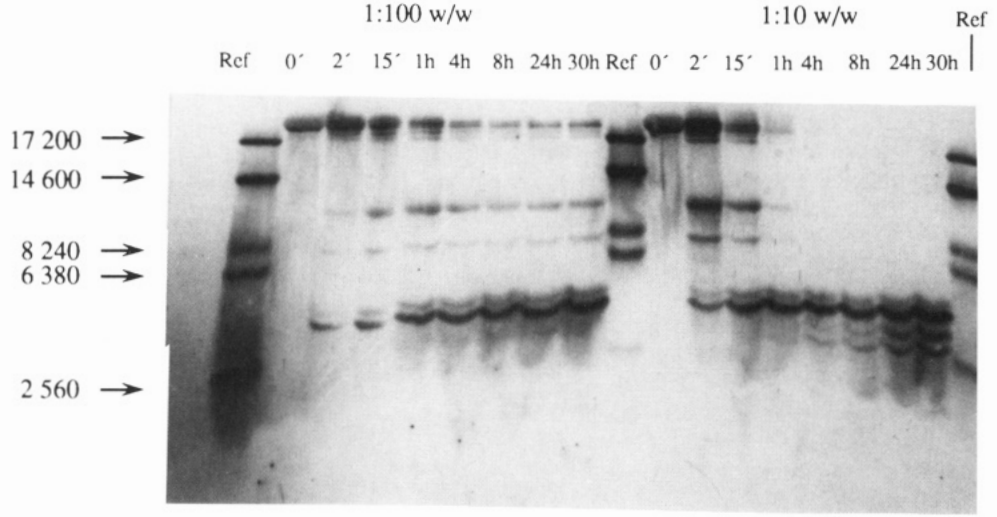
Determination of the Sites of Proteolytic Cleavage by Mass Spectrometry and Peptide Sequencing after Delipidation. Delipidation on the LH60 column does not only remove the lipids but also fractionates the peptides according to their size (Figure 5). Although each 500-μL fraction contains a mixture of different peptides, analysis by ES-MS was sufficient to describe their composition (Tables I and II of the supplementary materials). Otherwise, some peptides were further sequenced to remove any ambiguity (Table III of the supplementary material). Table IV of the supplementary material shows the molecular weights of proteolytic fragments deduced by the ES-MS spectrum in comparison with the molecular weights calculated from the sequence of thA. It shows that M_w values from ES-MS are within 0.5 of the theoretical ones. Every fragment had the specific sites for these enzymes at both termini except for two chymotryptic fragments (discussed later).

An alternative way to determine the cleavage sites by the proteases is to separate the peptides by SDS–PAGE and sequence them after transfer from gels onto solid support (see Materials and Methods). The results are shown in Table V of the Supplementary Material. They confirm the ES-MS results. But due to the uncertainty of molecular weight determination from SDS–PAGE peptide mobilities and the lack of efficient transfer of many peptides, only an approximation of the C-terminal end of the peptides could be estimated and only a few of the cleavage sites could be determined. Thus, this method provides far less information than the ES-MS method.

Limited Digestion with Trypsin. ES-MS analysis of the individual fractions eluted from the LH60 column shows that they contain more than one fragment (Table I, supplementary material). The elution peaks from the LH60 column overlap and therefore make it difficult to quantify each fragment precisely. Within each fraction, fragments were classified into four classes according to their relative amount: +++, major component of the fraction; ++, intermediate amount; +, small but significant amount; and +/−, probably present though not sure. For most of the fragments, the changes in concentrations of the peptides along the elution profile correlate with the molecular sieving property of the column, with the larger peptides eluting first.

The major tryptic cleavage sites are K4/A5, K51/T52, R54/S55, K73/I74, K113/V114, and K116/V117. With respect to the secondary structure of thA, the helical pair helices 1 and 2, helix 3, and helices 4 and 5 remained untouched by trypsin despite the presence of putative tryptic sites (Figure





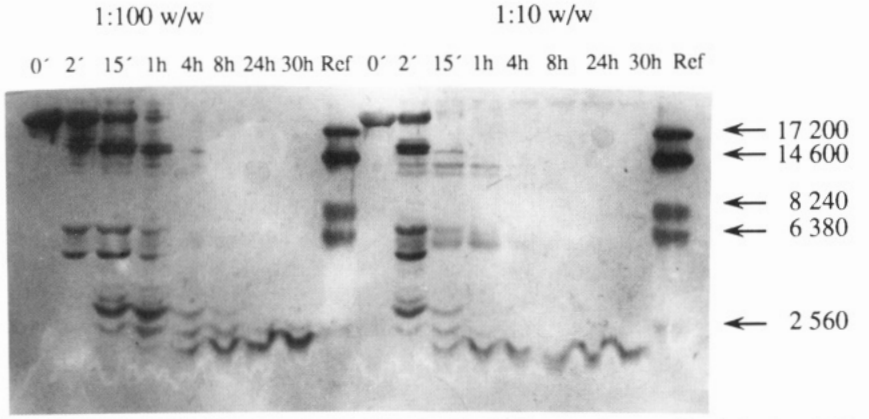


FIGURE 3: Kinetics of cleavage of the pore-forming domain of colicin A. (top) Cleavage by trypsin of the domain in solution at a protease/peptide ratio of 1/100 (left) or 1/10 (right) w/w. (middle) Cleavage by trypsin of the peptide bound to DMPG vesicles at a lipid/protein ratio R_i of 100/1 mol/mol. (bottom) Cleavage by α-chymotrypsin of the peptide bound to DMPG vesicles. In solution, α-chymotrypsin is unable to cleave the pore-forming domain of colicin A (not shown).

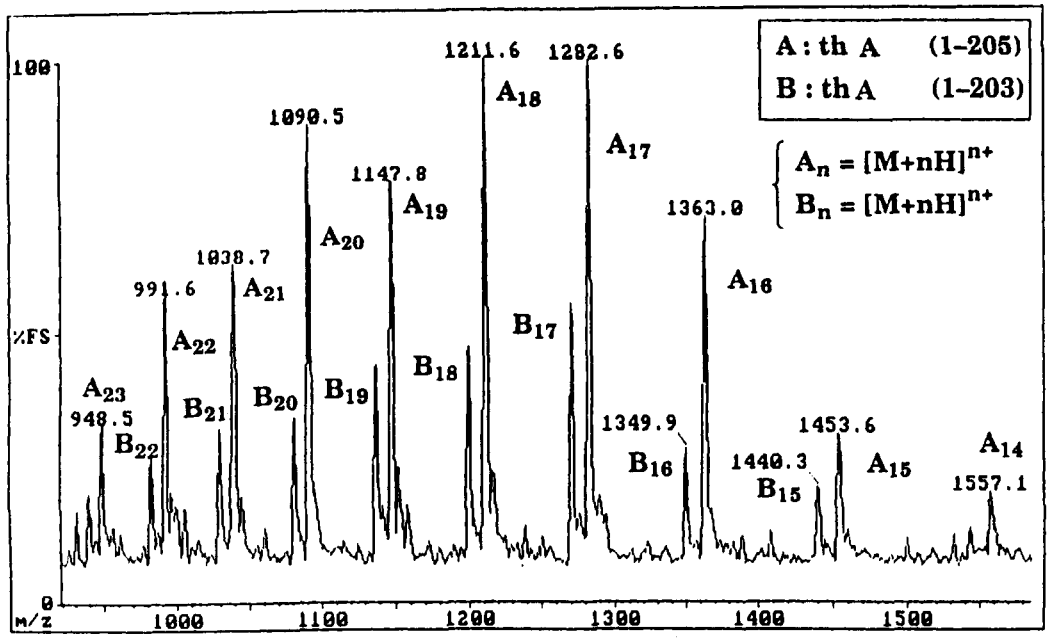


FIGURE 4: ES-MS spectrum of the intact thermolytic fragment (thA) of colicin A. The A peaks correspond to the intact thA peptide. The B peaks correspond to a thA peptide lacking two C-terminal amino acids.

Table I: M_w Values of the thCA31 Fragment of Colicin A Determined by ES-MS

component	no treatment	after delipidation	theoretical value[a]
major (1–205)	21 790.08 ± 1.96	21 795.61 ± 2.53	21 790.23
minor (1–203)	21 582.64 ± 1.12	21 588.08 ± 2.48	21 582.01

[a] Theoretical values were calculated from the amino acid sequence of thA.

6). The long sequence containing helices 7–10 also remained intact, in agreement with the presence of only one tryptic site (K190/F191 in helix 10). On the other hand, some hinge regions connecting two helices (2/3, 3/4) and the N-terminus are highly sensitive to tryptic attack. Not too surprising is the strong sensitivity of helix 6, because its sequence is extremely rich in basic residues and thus susceptible to trypsin digestion. However, comparison of the kinetics from Figure 3 (top and middle) indicates that in the soluble structure of the pore-forming domain this helix is much more resistant.

Limited Digestion with α-Chymotrypsin. The chymotryptic cleavage was analyzed and the fragment classified in the same way (Tables II and IVB, supplementary Material; Figure 7). In contrast to trypsin, there are two fragments, the M_w values of which predict a cleavage site which is less typical according to chymotrypsin specificity. Sequencing by Edman degradation reveals that the two fragments begin at V1 (Table III, supplementary Material), and they were indentified as V1-N64 and V1-N75. Interestingly, the two fragments were derived from the same unusual cleavage between N and K. It is known that chymotrypsin is able to cleave after aspargine and that the cleavage rate is enhanced by lysine as the adjacent residue (Konisberg & Steinman, 1977).

The major cleavage sites were N75/K76, W86/K87, F154/S155, and Y161/A162 (Figure 7). As observed with trypsin, the helical pair helices 1 and 2 and other helices (5 and 7) are preserved, whereas the hinge region between helices 3 and 4 is highly susceptible to proteolytic digestion. In contrast to trypsin, helix 6 remains intact, and one cleavage occurs in helix 10.

The most surprising feature of the cleavage pattern is the accessibility to chymotrypsin of helix 8 from the hydrophobic hairpin with two major and early cleavage sites after F154 and Y161.

DISCUSSION

Analysis of Proteolytic Peptides by ES-MS. In this study, electrospray mass spectrometry was used to identify the proteolytic fragments from thA. By ES-MS, it is possible to determine the molecular weight values of multicharged molecules such as proteins with a good precision (Covey et al., 1988; Loo et al., 1990; Mann et al., 1989; Smith et al., 1990; Van Dorsselaer et al., 1990). As shown in Tables IVA and IVB in the supplementary Material, the molecular weights of the entire thA fragment and its degradation products were determined with uncertainties lower than 0.04%. Furthermore, except for a few cases, the corresponding sequences were uniquely identified (Tables I and II, supplementary material) by searching along the total sequence of the thCA31 fragment stored on a personal computer without the help of N-terminal sequencing. These results demonstrate that even with fractions containing several peptides ES-MS is an excellent and efficient technique for the identification of proteolytic fragments.

Kinetics of Digestion by Trypsin and α-Chymotrypsin with and without Lipids. Comparison of the kinetics of digestion by trypsin (or α-chymotrypsin) of the pore-forming fragment (thA) in solution and bound to DMPG vesicles (Figure 3) shows a profound difference of sensitivity between the two forms of the peptide. It implies that the membrane-bound structure is more loosely packed than the soluble form. It was shown that the α-helical content of both conformations is the same but that aromatic side chains are much more mobile in the membrane-bound state (Lakey et al., 1991b). Furthermore Muga et al. (1993) have shown that the membrane-bound state is also in a molten globule-state conformation. The fact that cleavage sites occur at some hot spots and are not spread entirely along the sequence and correspond mainly to loops or hinge regions on the protein X-ray structure (see discussion below) confirms that secondary structure elements are preserved in the membrane-bound state despite the loss of tertiary structure.

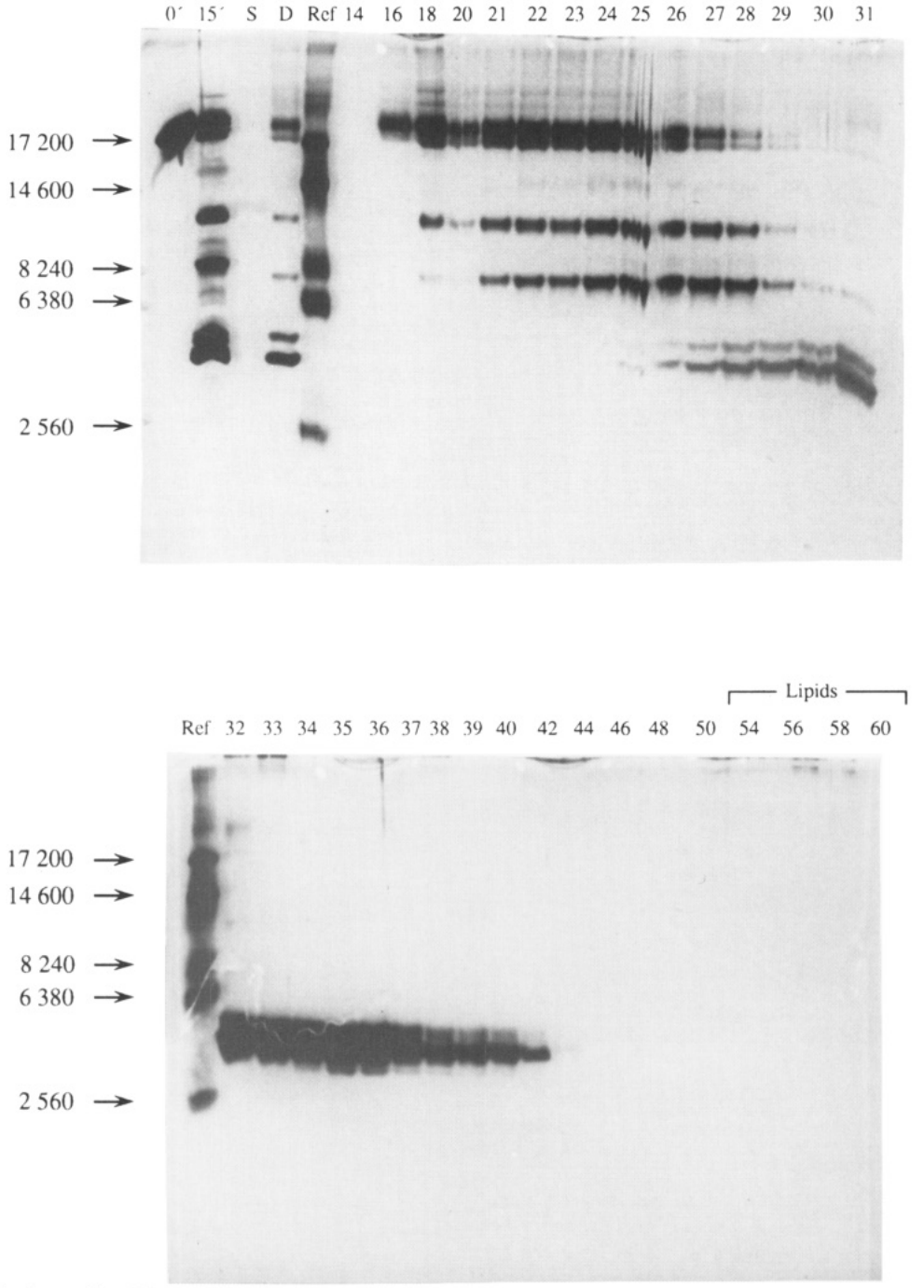


FIGURE 5: LH60 elution profile of the tryptic digest of thA bound to DMPG after solubilization in $CHCl_3$/MeOH 1/1 adjusted to pH 4 with 1 M HCl. The digestion products are sorted by molecular weight and delipidated.

Sites Accessible to Trypsin and α-Chymotrypsin in the Structure of the Membrane-Bound Form of the Pore-Forming Domain of Colicin A. Different models have been proposed for the membrane-bound structure of the pore-forming domain of colicin A (Parker et al., 1989, 1992; Lakey et al., 1993) (Figure 1). Although this has not yet been fully demonstrated, one of the basic features of these models appears to be the conservation of the helices between the structure in solution and that in the membrane. We therefore shall discuss the cleavage patterns by trypsin and chymotrypsin in light of the known X-ray structure of the domain (Parker et al., 1989, 1992).

The specificities of the two proteases are very different. Trypsin cleaves after basic residues, whereas chymotrypsin cleaves essentially after aromatic and hydrophobic residues with a few exceptions (see Results). Therefore, a direct comparison of the cleavage patterns is not an easy task. Moreover, charged groups are known to be mostly localized at the surface of proteins, whereas hydrophobic residues are predominantly localized in the core of the structures. Moreover, in the model of amphipathic helices lying parallel to the membrane surface, hydrophobic residues are believed to be buried in the hydrophobic core of the bilayer. This could explain the higher number of cleavage sites observed with trypsin than with α-chymotrypsin. However, some conserved features of the cleavage pattern by the two enzymes are worth mentioning and could be interpreted in terms of accessibility.

First, the observed cleavage sites are not spread uniformly over the sequence but are localized at some hot spots, some of them being shared by both enzymes (Figures 6 and 7). The helical hairpin 1/2 and helices 5 and 7 are not hit by the proteases. The loop between helices 3 and 4 around K73 is

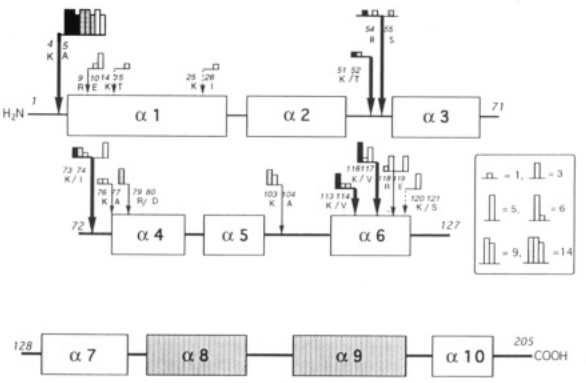


FIGURE 6: Schematic representation of trypsin cleavage sites of the membrane-bound thA. Locations of the α-helices are indicated by boxes. The hydrophobic hairpin (helices 8 and 9) is shaded. The thickness of the arrows pointing to the cleavage site is proportional to the amount of peptide found with the following correspondence with Table I of the supplementary material: thick/+++, thin/++, and dashed/+. The horizontal extension of the arrows points to the right or to the left to indicate an N-terminal or C-terminal end, respectively. The histogram represents the frequency of the cleavage at the site: closed/+++, dotted/++, and open/+.

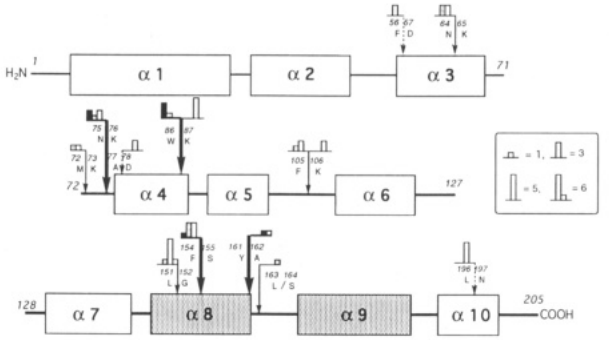


FIGURE 7: Schematic representation of α-chymotryptic cleavage sites of the membrane-bound thA. Same representation as Figure 6. See also correspondence with Table II of the supplementary material.

extremely accessible to both proteases. Apart from one hot spot localized on helix 6 for trypsin and one on helix 8 for α-chymotrypsin which will be discussed below, the major cleavages occur in loops. This supports spectroscopic evidence from circular dichroism and infrared spectroscoy (Goormaghtigh et al., 1991; Rath et al., 1991; Lakey et al., 1991b) that the two forms of the pore-forming domain of colicin A share the same elements of secondary structure.

Combining the data from this study with those from fluorescence studies on labeled single Cys mutants of colicin A (Lakey et al., 1991a, 1993) and chemical modifications of histidines (Massotte, 1991), it becomes clear that all the amphipatic helices (1–7 and 10) lie at the surface of the membrane.

More difficult to interpret is the high sensitivity of helix 6 to trypsin. The hydrophilic face of helix 6 is rich in basic residues, and at first glance it is not surprising to find tryptic cuts there. However, this region becomes clearly more sensitive when the domain is bound to the membrane. In the crystal structure, helix 6 forms a helical pair in intimate and extensive contact with helix 7 (Parker et al., 1992). This interaction may be lost or helix 6 may become distorded upon membrane insertion and reorganization of the neighboring helix 5 as predicted by Parker et al. (1992). Unfortunately, helix 7 does not contain putative tryptic sites, and it is also difficult to interpret the lack of α-chymotryptic cleavage on this helix.

The Hydrophobic Hairpin Is Accessible to Proteases. The most surprising and remarkable result of this study is the early and efficient cleavage by α-chymotrypsin of the hydrophobic hairpin at the level of helix 8, mainly at F154/S155 and Y161/162 (Tables II and IVB, supplementary material; Figure 7). From these observations, it could be concluded that the hydrophobic hairpin spends a significant time at the membrane surface with helix 8 accessible to proteolytic attack. However, in the soluble structure of thA, Y161 (on helix 8) interacts with W86 (on helix 4) at a distance of 3.57 Å according to the X-ray structure. W86 in helix 4 is the site of an early cleavage by chymotrypsin. Therefore, another explanation could be that first cleavage occurs at W86 on helix 4, which would unmask Y161, which could as in the soluble structure be in contact with W86. Cleavage at Y161 on helix 8 may thus occur only as a consequence of cleavage at W86. The absence of a chymotryptic fragment V1-Y161 in the digestion mixture and the appearance of the chymotryptic peptide A162-H205 only after 5 min of digestion by the protease (Figure 3, bottom, 4264 MW peptide and data not shown) support this hypothesis. This interpretation does not rule out a parallel orientation of the hydrophobic hairpin within the membrane plane but suggests that the hairpin may not be fully accessible to solvent in the uncleaved thA. On the other hand, cleavage after F154 occurs already after 2 min of incubation with α-chymotrypsin (Figure 3, bottom, 16 880 MW peptide and sequencing data not shown), and the major resulting peptide A1-F154 contains an intact N-terminus. These results suggest strongly that helix 8 is directly accessible to the protease. These data support the penknife model (Figure 1C) rather than the umbrella model (Figure 1B) as suggested in recent studies (Duché et al., 1993; Lakey et al., 1991a, 1993). In agreement with these data, we were unable to detect any parts of the thA peptide chain protruding on the inside of the vesicles with either trypsin, chymotrypsin, or pepsin trapped inside the vesicles (data not shown; Massotte, 1991).

In a recent study, Zhang and Cramer (1992) probed the accessibility of the homologous colicin E1 to proteases. In agreement with this study, there is a similar cleavage site in the loop between helices 2 and 3 by trypsin in the colicin E1 C-terminal peptide bound to asolectin liposomes, and proteinase K cleaves also the peptide chain between helices 4 and 5. The absence of cleavage by trypsin of helix 6 may be explained by the lack of lysine and arginine residues on that part of the colicin E1 sequence. There are, however, major differences between the two studies. Colicin E1 was found much more resistant to protease degradation than colicin A, and no cleavage could be observed at the level of helix 8 despite the presence of a phenylalanine residue in colicin E1 at the equivalent position of F154 in colicin A. There are several explanations which can be put forward to explain these apparent contradictions. The major one is that the colicin E1 study was carried out at pH 4.0, a pH at which trypsin and α-chymotrypsin are almost inactive. This is probably why long incubation times, were necessary to observe some cleavage by the proteases. We cannot, however, preclude that both colicins are more protected by the lipids at acidic pH from proteolytic degradation than at pH 7.0, the pH used in this study. Nevertheless, the disulfide bond engineering study by Duché et al (1993) and the "site-directed fluorescence" experiments by Lakey et al. (1993) were carried out at pH 5.0. Both studies suggest also a parallel orientation of the hydrophobic hairpin of the membrane bound colicin A at acidic pH.

In summary, we confirm that, in the absence of a membrane potential, the pore-forming domain of colicin A lies at the

surface of the membrane in a looser conformation than in its soluble state but with the same secondary elements. Orientation of the hydrophobic hairpin perpendicular to the plane of the membrane seems not to be the preferred conformation.

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SUPPLEMENTARY MATERIAL AVAILABLE

Extensive information on the composition of the fractions eluting from the LH60 columns after trypsin and α-chymotrypsin digestion, experimental and theoretical molecular weight of the peptides, and N-terminal sequencing of some peptides (6 pages). Ordering information is given on any current masthead page.

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