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Characterization of domain borders and of a naturally occurring major fragment of staphylococcal α-toxin

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A naturally occurring staphylococcal α-toxin fragment with an apparent membrane-binding capacity but without toxic activities is shown to be derived from the C-terminal half of the intact polypeptide chain by cleavage between position 134 and 135 in the parent molecule. The resulting N-terminus is slightly ragged with a fragment start not only at position 135 but also at the adjacent position 136. Another naturally occurring fragment starts at position 9, derived from an original cleavage between position 8 and 9 in the parent molecule. Analysis of non-purified fragment mixtures confirmed these positions and established that only one further region, at positions 71–72, is partly sensitive to proteolysis under natural conditions. Trypsin treatment has limited effects on the native toxin molecule, giving essentially only two initial cleavages with resultant large fragments. One of these cleavages is at the peptide bond between position 131 and 132, thus only three residues away from the position of the major naturally occurring cleavage. The other bond sensitive to trypsin is between position 8 and 9, thus identically positioned to the cleavage occurring naturally. Together, all the cleavages define a region in a central segment of the polypeptide chain that has all the properties of an inter-domain segment. The C-terminal half appears to constitute a membrane-binding domain, and the N-terminal half a structure needed for full biological activity, functionally subdividing the parent polypeptide chain.

Staphyloccocal α-toxin; Native fragment; Membrane binding; Tryptic cleavage; Electroblotting; Amino acid sequence analysis

1. INTRODUCTION

Different clinical isolates of *Staphylococcus aureus* secrete various membrane damaging toxins, of which the α-toxin is considered to be the major virulence factor [1]. α-Toxin is a single polypeptide (M_r 33000) with lethal, dermonecrotic, cytotoxic and hemolytic properties, all probably dependent on the membrane damaging primary action of the toxin [2,3].

At high concentrations, α-toxin forms hexameric circular structures on artificial and natural membranes [4]. It has been postulated that hexameric α-toxin forms transmembrane pores [5], and in rabbit red blood cell membranes, part of the α-toxin molecule has been suggested to be embedded within the hydrophobic region [6]. A model for the toxin assembly has been proposed [7], in which the monomer is suggested to consist of two domains linked by a hinge region; upon interaction with membranes the hinge region opens up exposing occluded hydrophobic surfaces. The hydrophilic monomer is thereby converted to an amphipathic rod that becomes a subunit of the transmembrane hexameric pore [7]. The α-toxin gene has also been cloned and the DNA and amino acid sequences have been reported [8].

The biological properties of a naturally occurring fragment of α-toxin have been described [9]. The fragment lacks the lethal and cytotoxic ac-

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tivities but inhibits these effects of the native toxin, suggesting that the membrane-binding region of α-toxin is contained in the fragment, but that a region required for the toxic effects of α-toxin is outside this fragment.

In the present study, we have characterized the cleavage site producing this fragment and another naturally occurring minor fragment. Similarly, trypsin was found to give highly specific and limited cleavages of the native toxin. Amino acid sequence analysis of the naturally occurring fragments and the tryptic fragments showed that major cleavages are in a short segment at the center of the polypeptide, in an N-terminal part, and in between these two regions. The data support a model for the toxin based on the existence of two domains and define the domain borders. Moreover, the cell binding activity can be attributed to one of these domains in agreement with previous suggestions of separate functional parts [3,7,10].

2. MATERIALS AND METHODS

2.1. *Preparation of toxin and fragments*

α-Toxin was prepared from *S. aureus* (strain Wood 46) and purified as described [11].

A naturally occurring fragment (apparent M_r 18500) was obtained from an apparently normal α-toxin purification (fig.1) [9]. This fragment (here designated NF-I) lacked the lethal and cytotoxic activities of the intact α-toxin. However, it was neutralized by polyclonal anti-α-toxin and retained the membrane-binding site since it inhibited the effects of native α-toxin [9]. The same purification also yielded small amounts of a smaller (apparent M_r 14000) fragment (designated NF-II).

Tryptic digestion of native α-toxin (1 mg in 1.5 ml Tris buffered saline) was performed for 4 h at 37°C with 30 μg trypsin at pH 8.0. The reaction was terminated by the addition of 30 μg lima bean trypsin inhibitor. These conditions were found to be optimal for production of two stable fragments (TF-I and TF-II) according to SDS-polyacrylamide gel electrophoresis (fig.1) [6,12].

2.2. *Amino acid sequence analysis*

The four polypeptide fragments (400–500 pmol) purified by SDS-polyacrylamide gel electrophoresis were detected by soaking the gel in 1 M potassium chloride, which made protein bands temporarily visible as clear zones against an opaque background (modified from [13] by omission of acetic acid). Appropriate bands were excised and electroblotted onto Polybrene-impregnated glass fiber filter discs using an ISCO model 1750 electrophoretic sample concentrator in a novel application [14]. The filter discs with the separately transferred and immobilized fragments were then subjected to degradations in an Applied Biosystems 470A gas-phase sequencer.

The non-separated preparations were transferred to 30% acetic acid by dialysis in Spectrapor 3 tubing (native fragments), or by Sephadex G-10 chromatography (tryptic fragments). They were then degraded directly in a Beckman 890D liquid-phase sequencer, after application into glycine-precycled Polybrene, as described [15].

3. RESULTS

3.1. *Characterization of the cleavages yielding the naturally occurring fragments*

One major naturally occurring fragment (NF-I) of α-toxin has an apparent M_r of about 18500 (fig.1). Sequence analysis of this fragment revealed that it is largely homogeneous but has a ragged end, starting with Leu-135 (about 80% of all molecules) or Ile-136 (about 20%). The results (table 1) prove that this fragment is derived from cleavages at two adjacent bonds in the center of the parent α-toxin molecule. The calculated M_r of a fragment starting at Leu-135/Ile-136 and ending at the original C-terminus would be 18476/18363, which is in good agreement with the value 18500 estimated from SDS-polyacrylamide gel electrophoresis (fig.1). It may be concluded that this naturally occurring α-toxin fragment, which contains the binding activity, corresponds to a domain formed by the C-terminal half of the parent molecule.

Another reproducibly obtained fragment, NF-II (fig.1), yielded a homogeneous N-terminal sequence (table 1) which can be similarly identified as starting at Thr-9 in the parent molecule originally secreted [8]. In order to examine the possible existence of still further fragments, the original preparation was also analyzed without fragment separation by application to a liquid-phase sequencer. The results show the presence of the same

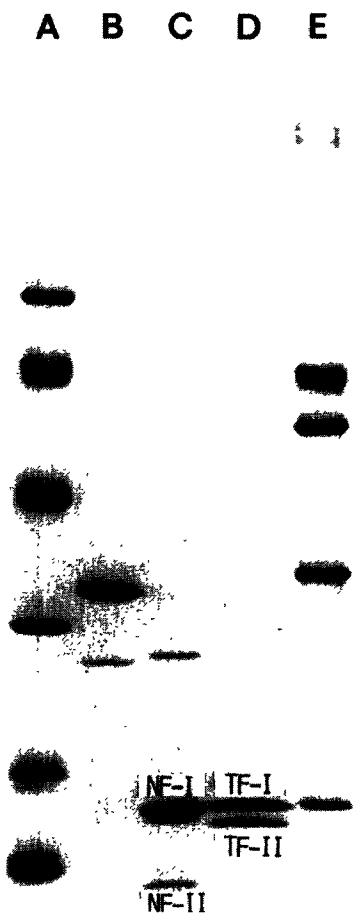

Fig.1. SDS-polyacrylamide gel electrophoresis of the fragments analyzed. Lanes A and E are marker proteins (phosphorylase *b*, serum albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor, α-lactalbumin in A; thyroglobulin, serum albumin, catalase, lactate dehydrogenase, ferritin in E). The intact α-toxin is separated in lane B, the naturally occurring fragments in lane C, and the tryptic fragments in lane D.

two sequences as from the purified NF-I and NF-II fragments, thus with structures starting at Leu-135 and Thr-9, respectively. Relative yields of these two fragments in the sequencer degradations of the intact mixture were in a ratio of 1:0.4. (The fragment corresponding to Ala 1–Lys 8 was not observed but was conceivably removed in the dialysis step prior to the application to the sequencer.) In addition, a third sequence was observed, corresponding to a start at Gly-72 (NF-III), also in a lower yield, about 0.3. Finally, the minor sequence of NF-I (starting at Ile-136) could possibly be traced but was less than 0.1 of the total. Together these structures account for all residues observed in the degradation of the unseparated material.

Thus, as summarized in fig.2, it can be concluded that the naturally occurring fragments are derived essentially from cleavages at only three regions. They are at peptide bonds Gly-134–Leu-135 (high yield, presumably corresponding to stoichiometic cleavage), and at peptide bonds Lys-8–Thr-9 and Glu-71–Gly-72 (both in about half the major yield, thus together approaching the NF-I-stoichiometry). Even considering the minor cleavages, specificities are extensive, since only one other site was found to be partly sensitive, and this is the bond Leu-135–Ile-136 adjacent to the major cleavage point.

3.2. *Characterization of the tryptic cleavage products*

Treatment of intact α-toxin with trypsin produced two (apparent M_r 18000) fragments (TF-I and TF-II, fig.1), suggesting that accessibility to tryptic cleavage is highly limited in the native molecule. Gas-phase sequencer analysis revealed that fragment TF-I is a homogeneous product, starting at Ile-132 of the parent α-toxin (table 1). Thus, as shown in fig.2, trypsin cleaves the native toxin in the center of the molecule, at a peptide bond only three residues away from the cleavage site producing the major naturally occurring fragment NF-I with the binding activity. The calculated M_r of TF-I is 18703, in reasonable agreement with the observed value of ~18000. Similar analysis of TF-II revealed that it also is homogeneous, starting at position Thr-9 of the parent molecule (table 1). The apparent M_r of TF-II (~17000) is larger than the one calculated from the amino acid sequence, 13446, provided TF-II ends at Lys-131. However, this deviation is within the possible range of the estimates from the electrophoretic migration. Furthermore, it may be noticed that the apparent M_r of TF-II from electrophoresis is larger than that of NF-II (fig.1) as expected from the relative positions of the characterized cleavage sites (fig.2).

As in the case of the preparation containing the naturally occurring fragments (above), a tryptic digest of the native molecule was also submitted to

Table 1

Results of gas-phase sequencer degradations of the different fragments that were analyzed after electroblotting

Naturally occurring fragments		Tryptic fragments	
NF-I	NF-II	TF-I	TF-II
1. Leu 133 Ile 42	1. Thr 59	1. Ile 52	1. Thr 45
2. Ile 126 Gly 45	2. Gly 80	2. Gly 40	2. Gly 38
3. Gly 123 Ala 34	3. Thr 53	3. Gly 34	3. Thr 33
4. Ala 114 Asn 37	4. Thr 41	4. Leu 37	4. Thr 30
5. Asn 99 Val 37	5. Asp 51	5. Ile 30	5. Asp 35
6. Val 111 Ser 19	6. Ile 40	6. Gly 27	6. Ile 40
7. Ser 55 Ile 22	7. Gly 31	7. Ala 27	7. Gly 25
8. Ile 106 Gly 15	8. Ser +	8. Asn +	8. Ser 15
9. Gly 78	9. Asn 21	9. Val 22	9. Asn +
	10. Thr 13		

Values show pmols recovered in each cycle. Samples were purified by SDS-polyacrylamide gel electrophoresis and transferred to glass fiber filter discs as described [14]

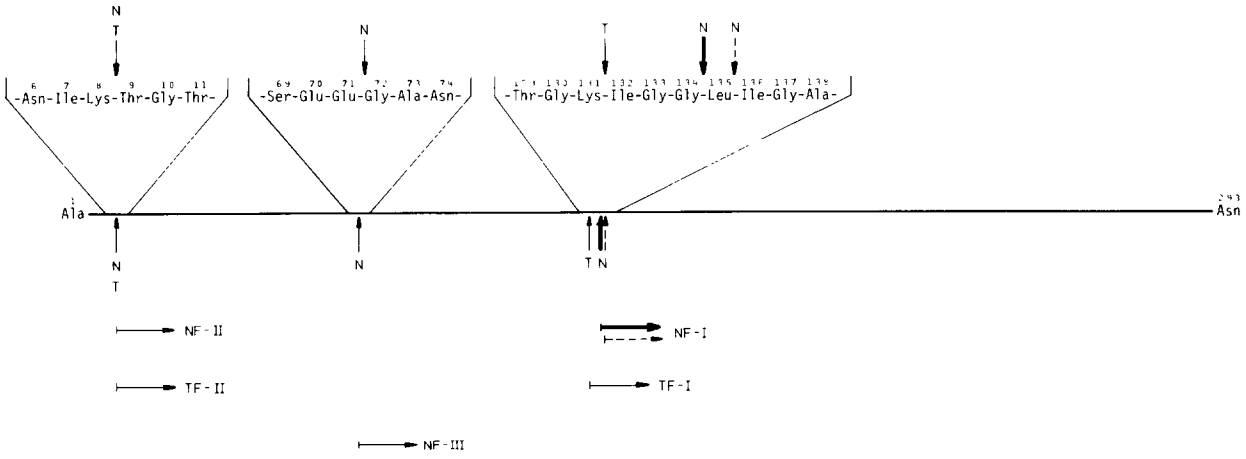

Fig.2. Schematic diagram showing the relative positions of the cleavage sites defined. The continuous line shows relative positions of the regions discussed in the mature α-toxin structure, drawn to scale and derived from [8]. Relevant portions now analyzed in the cleavage products are enlarged above the continuous line, with data from table 1. T, tryptic cleavage, N, native cleavage, at positions indicated by the arrows: thick arrow, major cleavage in high yield; thin arrow, a major cleavage; dashed arrow, a minor cleavage. Results of the sequencer degradations of the isolated fragments and the unseparated mixtures are indicated below. Both TF-II and NF-II suggest smaller sizes than those estimated from apparent electrophoretic mobilities (fig.1). Consequently, C-terminal regions of these fragments are only tentative. The absolute migrations of TF-II and NF-II (fig.1) are small in relation to that expected from the sizes concluded from the characterized cleavage points. However, the relative migration differences between TF-II and NF-II (fig.1) are consistent with the relative size differences suggested from the cleavage points.

direct sequencer analysis without prior peptide purification, in order to judge relative ratios of all cleavages. The results showed essentially three major N-terminal sequences, starting at positions Ala-1, Thr-9, and Ile-132. However, the degradation results from the tryptic digest were far from as conclusive as those from the preparation of the naturally occurring fragments (above), and also showed minor cleavages at several other positions that could not be fully interpreted in detail. Consequently, trypsin is concluded to have a stronger and further action on the entire polypeptide chain than the protease(s) giving the naturally occurring fragments. Significantly, the electrophoretically observed tryptic fragments (fig.1) gradually disappear on extended digestions, in agreement with the results from sequence analysis. In any event, the analysis of the unseparated digest confirms the results from analysis of the tryptic fragments (table 1) and defines the regions of initial and maximal sensitivity to tryptic cleavage.

4. DISCUSSION

Based on previous characterizations of the naturally occurring fragment (NF-I) a hypothetical map of α-toxin was constructed, in which the binding region of α-toxin was placed in the center of the molecule [9]. The binding fragment was then thought to correspond to the N-terminal half of the parent molecule, based on comparison with two previously described biologically active tryptic fragments [16]. However, the sequence analysis now locates the N-terminus of the binding fragment at positions 135/136, indicating that this fragment in fact corresponds to the C-terminal half of the parent molecule.

Sequence analysis of the two fragments obtained by tryptic digestion of α-toxin placed their N-terminal regions very close to or identical with those of the naturally occurring fragments (fig.2), thus confirming that native α-toxin is accessible to mild proteolytic digestion in the middle of the polypeptide chain and to a somewhat lower extent only at two regions in the N-terminal half. The cleavage region at the center of the molecule coincides with a postulated inter-domain hinge region [7], based on digestion with proteinase K. This is also one of the three high hydrophobicity regions of α-toxin [8]. The membrane-binding site of α-toxin may be located near the N-terminus of NF-I, since the C-terminal CNBr-fragment has been shown before to be non-essential for binding [10].

We have not been able to detect any separate biological activity in the fragments starting at Thr-9. However, it was inferred from the recent characterization of CNBr-fragments of α-toxin [10] that this half of the molecule may be essential for adequate hexamer formation, as also implicit in the postulated assembly model [7].

In summary, the present results establish a two-domain structure for α-toxin. Naturally occurring fragments, and those from tryptic treatment give essentially similar cleavage patterns, with a central accessible region which has been defined at positions between 131 and 135 in the native molecule. The results indicate that the binding region is situated in the C-terminal domain and that full biological activity also requires the N-terminal domain.

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