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CIRCULAR DICHROISM AND STRUCTURE- FUNCTION RELATIONSHIPS IN CLOACIN DF13- IMMUNITY PROTEIN COMPLEX

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SUMMARY: Comparison of the circular dichroism (CD),of cloacin-immunity protein complex with that of cloacin and of a mutant cloacin lacking the ability to bind immunity protein, shows that the binding of immunity protein imposes a definite structure on the cloacin molecule. It is discussed that this structure probably is a prerequisite for an effective killing activity of the bacteriocin. The cloacin molecule itself probably has two domains, as was found by limited proteolysis. Comparison of the structure of two of the proteolytic fragments with that of the intact molecule by means of circular dichroism also suggests that cloacin is made up of a part without much periodic structure and of a part with more helicity. The former part being rather sensitive to proteolysis, the latter being comparatively insensitive.

INTRODUCTION: Cloacin DF13 is a bacteriocin produced by cells of Enterobacter cloacae, harbouring the bacteriocinogenic plasmid Clo DF13 (1). The bacteriocin is excreted as an equimolar complex of the actual cloacin (molecular weight 56.000) and its immunity protein (molecular weight 10.000) (2). The killing of sensitive cells by the bacteriocin is the result of two events: (a) enzymic inactivation of the ribosomes by a specific endoribonucleolytic cleavage of the 16 S ribosomal RNA and (b) a leakage of potassium ions across the cytoplasmic membrane (3, 4).

The immunity protein inhibits the enzymatic activity of cloacin, thereby giving 'immunity' to the cloacinogenic cells (5). It was however discussed by Oudega et al (6) that the binding of immunity

Througout this paper cloacin complex stands for the complex of cloacin and immunity protein, whereas cloacin stands for the cloacin moiety of this complex.

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protein also assists the cloacin molecule to adopt a structure, necessary for the penetration of the outer membrane of sensitive bacteria. In order to obtain additional evidence for this role of the immunity protein, circular dichroism measurements were performed to compare the structure of cloacin complex, cloacin and immunity protein. The measurement of the structure of a mutant cloacin shortened at the C-terminal and lacking the capacity to bind immunity protein (7), confirmed our observations. On the basis of amino acid analysis of cloacin fragments de Graaf et al (8) proposed that the cloacin has a domain type structure. The N-terminal part of the cloacin molecule appeared to consist mainly of hydrophobic amino acid residues and is highly susceptible to proteolytic cleavage, suggesting the absence of much helicity. The hydrophilic C-terminal part of the molecule is rather resistant to proteolytic digestion and therefore probably has a more definite structure. Support for this suggestion could be obtained by comparing the CD spectra of two of these proteolytic fragments with that of the intact molecule.

MATERIALS AND METHODS: Bacteriocin preparations. Wild-type cloacin complex, cloacin and immunity protein were prepared as described (2, 9). Cloacin pJN82 was prepared as described for wild type cloacin. The two proteolytic fragments of cloacin were prepared as described by de Graaf et al (8). All proteins were stored at -20°C as lyophylisates. Solutions suitable for circular dichroism measurements (0.2-0.3 mg/ml) were prepared in 0.05 M potassium phosphate buffer pH 6.9, containing 0.2 M potassium fluoride. The solutions were used immediately after preparation.

Circular Dichroism Measuerement. CD measurements were performed on a Cary 61 spectropolarimeter in cells with a path length of 0.2 cm. The readings were performed at 10°C. All recordings were done with a scanning speed of 0.4 nm/sec and a pen sensitivity of 100. The ellipticity curves were drawn taking average values from several recordings and are expressed in terms of mean residue ellipticities in deg.cm^2 dmole^{-1}. The mean amino acid residue weights were calculated from the amino acid analyses of the different proteins. A value of 106.2 was used for cloacin-complex, 102.2 for cloacin and cloacin pJN82, 109.1 for the "thermolysin" and "thrombin" fragment, and 133.8 for the immunity protein. Analysis of the curves into percent α-helix, β-sheet and random coil structure was performed by the method of Chen et al (10).

Ultraviolet Absorbtion. Ultraviolet absorption spectra of the bacteriocin preparations were recorded on a Varian superscan or on a Zeiss-DMR21 spectrophotometer. The concentration of the bacteriocin preparations was calculated from the absorbance at 280 nm.

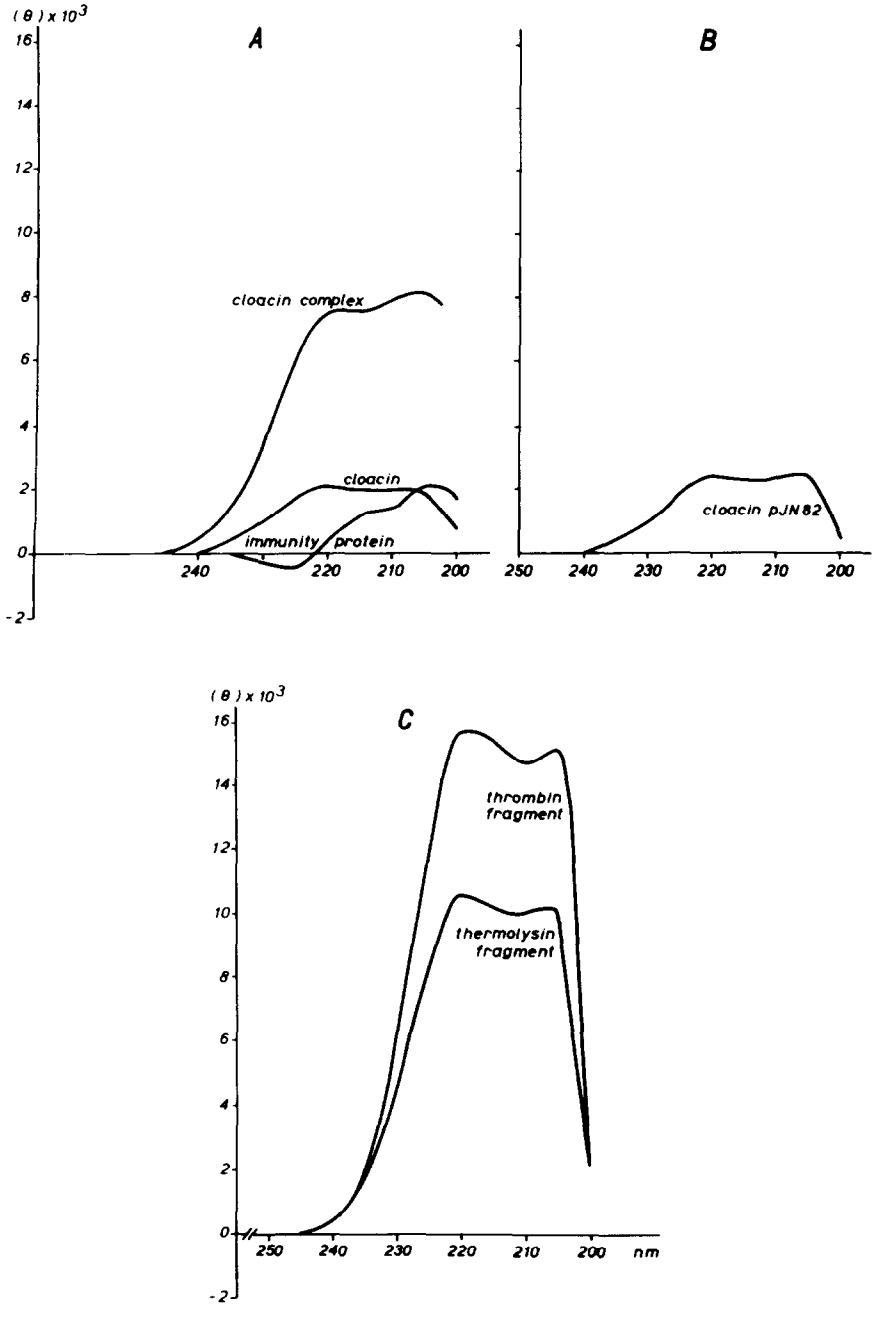

Figure 1. A. Circular Dichroism spectra of cloacin complex, cloacin and immunity protein at a concentration of 0.2-0.3 mg/ml in 0.05 M potassium phosphate buffer pH6.9, containing 0.2 M potassium fluoride. Measurements were performed at 10°C in cells with 0.2 mm path length. B. Circular Dicroism spectrum of cloacin pJN82. Circumstances see caption A. C. Circular Dichroism spectra of "thermolysin" and "thrombin" fragments of wild-type cloacin. Circumstances see caption A.

Table I.

Estimation of the percentage α-helix and β-sheet conformation in cloacin and cloacin derivatives from the amplitude of resolved CD curves by the method of Chen et al. (10)

	%α-helix	%β-structure	%random
Cloacin complex	22	24	54
Cloacin	2	51	47
Cloacin pJN82	3	50	47
"Thermolysin"fragment	31	22	47
"Thrombin"fragment	46	13	41
Immunity protein	0	55	45

RESULTS AND DISCUSSION: The CD spectra of wild-type cloacin complex, cloacin and immunity protein are given in Fig. 1A. Analysis of these spectra (10) gives the values for percentage α-helix, β-sheet and random coil structure (Table I). From these values it is clear that the binding of immunity protein to cloacin induces a conformational change in the structure of the cloacin molecule. The percentage α-helix i.e. is raised from 2% to 22% and the percentage β-sheet is reduced from approximately 50% to 25%.

A conformational change in the structure of cloacin molecules upon binding of immunity protein has also been demonstrated by changes in the fluorescence response of cloacin-bound 1-anilino-naphtalene-8-sulfonate (5). As suggested by Oudega et al (6), this change in conformational structure of the cloacin molecules upon binding of immunity protein may be the reason for the strongly reduced killing activity of cloacin compared to cloacin complex. To obtain additional evidence for this difference in conformation the CD spectrum of cloacin pJN82 has also been recorded. Cloacin pJN82 was isolated by Andreoli et al (11) and is shortened at the C-terminal by approximately ten amino acid residues. This mutant cloacin has lost the ability to bind immunity protein (7) and has no in vivo killing

activity (11). As shown in Fig. 1B, cloacin pJN82 has a CD spectrum comparable to that of wild-type cloacin giving a similar percentage of α-helix and β-sheet as in wild-type cloacin (Table I). These results therefore confirm the earlier observations that binding of immunity protein alters the conformation of the cloacin molecule, whereby the cloacin molecule probably adopts the right conformation for penetration of the outer membrane (6). It can be argued that the isolation of wild-type cloacin by dissociation of the cloacin complex with denaturating agents influences the cloacin structure (reconstitution of the complex never restores 100% killing activity). The fact, however, that the CD spectra of cloacin and cloacin pJN82 (isolated without denaturating agents) resemble each other so closely makes it unlikely that the structure of wild-type cloacin is affected by remnants of denaturants.

To obtain more information about the proposed domain type structure of cloacin (8), the CD spectrum of intact wild-type cloacin (Fig. 1A) has been compared with that of two fragments obtained from cloacin by limited proteolysis with thrombin and thermolysin (Fig. 1C). The 'thermolysin' fragment has a molecular weight of 36.000 and the 'thrombin' fragment of 35.000. Both fragments are derived from the C-terminal part of the cloacin molecule and contain almost all of the charged residues present in the intact cloacin molecule (8). No stable fragment was obtained from the hydrophobic N-terminal part of the cloacin molecule. This suggests that the N-terminal part of cloacin is more susceptible to proteolysis than the C-terminal part probably as a result of the absence of much helicity. As can be seen in Table I, the relative amount in α-helix content increases upon removal of the hydrophobic N-terminal part of the intact molecule. Therefore it can be concluded that the resistance of the C-terminal part of the cloacin molecule to proteolytic cleavage is probably due

to the presence of more periodicity in that part. Likewise the susceptibility of the N-terminal part of cloacin is probably due to the absence of ordered structures. Binding of immunity protein to cloacin can protect the N-terminal part from proteolysis, giving rise to the difference in susceptibility to proteolytic degradation between cloacin complex and cloacin as observed by de Graaf et al (8). This makes it plausible that immunity protein also interacts with the N-terminal part of the cloacin molecule on which it imposes a more rigid structure and thereby diminishes the susceptibility to proteolysis.

The great difference between cloacin complex and cloacin in α-helix content may than be explained as follows. Removal of immunity protein from the complex results in a partial loss of structure of the entire molecule. The structure and thereby the resistance of the C-terminal part to proteolytic degradation is regained upon removal of the N-terminal part by proteolytic enzymes.

In conclusion it can be said that the CD measurements described here, showed a substantial change in the structure of the cloacin molecule upon binding of immunity protein in accordance with earlier observations (5). These measurements also add to the proposed domain type structure of the cloacin molecule namely a hydrophobic domain at the N-terminal part, containing little periodicity and highly susceptible to proteolytic degradation and a hydrophylic domain at the C-terminal of the molecule, with more conformational structure and thereby less susceptible to proteolytic attack.

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