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THE INFLUENCE OF CHEMICAL MODIFICATION ON THE BIOLOGICAL PROPERTIES OF CLOACIN DF13

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SUMMARY: The influence of chemical modification on the biological properties of the bacteriocin cloacin DF13 has been investigated. All chemical modifications resulted in the total loss of the ability of the bacteriocin to kill sensitive bacterial cells. The ability of the bacteriocin to bind to specific receptor sites on sensitive bacteria was affected by the modification of carboxyl groups with glycine ethyl ester (GEE) and by the oxidation of tryptophan residues with N-bromosuccinimide (NBS). The endoribonucleolytic activity of the bacteriocin was affected by nitration of tyrosine residues with tetranitromethane (TNM) or by the oxidation of tryptophan residues with NBS. Binding of immunity protein to the cloacin was not affected by either of these modifications.

INTRODUCTION: The mode of action of the bacteriocin cloacin DF13 has been studied extensively during the last decade (1-7). However, it was only recently that something became known about the molecular structure and the structure-function relationships of the molecule (8,9). In these studies it was shown that the cloacin is a protein with two domains. A first domain, at the N-terminal part of the molecule, contains mainly hydrophobic amino acid residues and is possibly involved in translocation of the cloacin molecule through the cell envelope.

A second domain at the C-terminus contains most of the hydrophylic amino acid residues and is involved in the endoribonucleolytic activity of cloacin, in the binding to specific receptor sites on sensitive cells and in the binding of immunity protein. The immunity protein is an inhibitor of the enzymic activity of cloacin. Cloacin and immunity protein are excreted by the cloacinogenic cells as an equimolar complex (10), thereby giving "immunity" to the cloacinogenic cells (4). In order to obtain information

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about the nature of the amino acid residues involved in the various features of the cloacin action like receptor binding, ribonuclease activity, and binding of immunity protein, the effect of several specific chemical modifications of the cloacin molecule on these functions was tested.

MATERIALS AND METHODS: Bacteriocin Preparations. The complex of cloacin and its inhibitor, the immunity protein, as well as cloacin free of inhibitor, were prepared as described (9,10).
Assays of Bacteriocin Activity. Determination of the killing activity of cloacin preparations on sensitive cells was performed as described by de Graaf et al. (1). The ribonuclease activity of the modified cloacins was tested as described (5). The receptor binding capacity of the different cloacin preparations was assayed as described by Oudega et al. (6).
Modification Reactions. Modification of tyrosine residues with tetranitromethane (TNM) was performed as described by van der Zee et al. (12). The proteins were incubated for 3 h at 25°C in the dark with a 60-fold molar excess of TNM in 0.05 M phosphate buffer (pH 7.0). Reduction of the TNM modified cloacin complex was performed by incubation for 30 min with a 6-fold excess of sodium dithionite at 25°C in 0.05 M phosphate buffer (pH 7.0) as described (13).
Oxidation of tryptophan residues with N-bromosuccinimide (NBS) was performed as described (14) with a 10-fold excess of NBS in 0.05 M acetate buffer (pH 6.0). Incubation took place for 30 min at 25°C.
Amidation of carboxyl groups was carried out as described by Hoare and Koshland (15). Cloacin preparations were dissolved in 0.02 M [^{14}C]glycine ethyl ester (spec. act. 3 mCi/mmol) at pH 4.5. After addition of 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide (final concentration 0.05 M), incubation was continued for 1 h at room termperature. The reaction was stopped by the addition of 1.0 M acetate buffer (pH 4.5).
Peptide Analysis. 6.0 mg of modified protein was incubated with trypsin (enzyme : substrate = 1 : 50 w/w) for 4 h at 37°C in 0.1 M sodium carbonate buffer (pH 8.1). The digest was lyophilized and submitted to high voltage paper electrophoresis on Whatman 3 MM paper at 2.5 KV and pH 6.5 (16). Subsequently the digest was submitted to descending paper chromatography in a direction perpendicular to the high voltage electrophoresis as described by Waley and Watson (17). Nitrated tyrosyl peptides were detected by dipping the paper in 5% (v/v) ammonia in acetone (12). Tryptophan containing peptides were detected by the method described by Easly (18).
Circular Dichroism Measurements. Circular dichroism measurements were performed on a Cary 61 spectropolarimeter as described (19). Analysis of the curves into percentage α-helix, β-sheet and random coil structure was performed by the method of Chen et al. (20).

RESULTS AND DISCUSSION. The results of the different tests on the modified cloacin preparations are given in Table I. It is evident that all modifications result in the loss of killing activity on sensitive cells. The same holds for the modifications of histidine and arginine residues, that are not mentioned in this paper since it was not possible to quantitate the number of modified residues.

TABLE I

THE EFFECT OF CHEMICAL MODIFICATIONS ON THE BIOLOGICAL ACTIVITIES OF CLOACIN PREPARATIONS

Preparation	Number of modified residues	In vivo killing activity	Receptor binding	RNAse* activity
Cloacin complex	-	100 %	+	100 %
Cloacin	-	5 %	+	100 %
TNM cloacin complex	1 tyrosine	0 %	+	60 %
TNM cloacin	6 tyrosines	0 %	-	60 %
Reduced TNM cloacin complex	1 tyrosine	0 %	-	25 %
NBS cloacin complex	5 tryptophans	0 %	-	40 %
NBS cloacin	7 tryptophans	0 %	-	0 %
GEE cloacin complex	4 carboxyl groups	0 %	-	100 %

* RNase activities are expressed in percentages of the activity of the unmodified cloacin preparations.

Modification of Tyrosine Residues. From the analysis of nitrotyrosine on the peptide maps it was clear that only one tyrosine residue is modified in the complex of cloacin and immunity protein. Five additional modified tyrosine residues are found in cloacin. Apparently the presence of immunity protein in cloacin complex protects the cloacin against modification. However, the ability to bind immunity protein is not affected. Likewise, the immunity protein protects the cloacin against proteolytic degradation (8). The modification of one tyrosine residue in cloacin complex does not affect the capability of the complex to bind to the specific receptor sites on sensitive cells (Table I). This is in contrast to TNM cloacin that no longer binds to these receptors. Apparently one or more of the additional modified tyrosine residues found in cloacin are involved in receptor binding activity. Both TNM cloacin complex and TNM cloacin have a diminished endoribonucleolytic activity. Comparison of the CD spectra of the modified cloacin preparations and their unmodified analogues did not show significant differences. Therefore the effect on the endoribonucleolytic activity must

be due to the presence of nitrotyrosine residues. This may indicate the involvement of a tyrosine residue in either the enzymic activity of cloacin or in the affinity for bacterial ribosomes. Additional evidence was obtained from an experiment in which a fragment of cloacin, obtained by limited trypsinolysis (11), was modified with TNM. In this fragment the same tyrosine residue as in cloacin complex is modified, also accompanied by a substantial loss of endoribonucleolytic activity.

In order to determine whether the lower pI of TNM cloacin complex is responsible for its diminished enzymic activity, TNM cloacin complex was reduced with sodium dithionite. However, on the contrary, this reduction of the nitrotyrosine residue to an aminotyrosine even further reduced the enzymic activity.

Modification of Tryptophan Residues. The reaction with N-bromosuccinimide (NBS) oxidized five tryptophan residues in cloacin complex and seven in cloacin, as determined by peptide analysis. Due to this oxidation both cloacin complex and cloacin no longer bind to the receptor sites on sensitive cells, which may, as in the case of TNM cloacin, indicate the involvement of hydrophobic interactions in receptor binding.

The inhibition of protein synthesis by NBS cloacin complex is only 40% of that of the unmodified complex, whereas that of NBS cloacin is completely absent. Therefore, as in the case of tyrosine modification, this may also indicate the involvement of hydrophobic interactions in the enzymic activity of cloacin. The number of modified residues in both preparations as well as their residual activity suggests the involvement of more than one tryptophan residue. It is clear that also in this case the presence of immunity protein protects the cloacin molecule from modification.

Modification of Carboxyl Groups. The modification of colicin E3 with a water soluble carbodiimide and [^{14}C] labelled glycine ethyl ester yielded a modified colicin in which the extent of label incorporation indicated the modification of a single carboxyl group (21). Modification of cloacin complex in the same

way yielded a modified protein in which the extent of label incorporation indicates the modification of two carboxyl groups. Peptide mapping of the labelled cloacin complex and subsequent autoradiography showed four labelled peptides. This indicates that four carboxyl groups must have been modified, albeit for only 50%.

As in the case of colicin E3, this modification leads to the failure of the cloacin complex derivative to bind to the receptor sites on sensitive cells, accompanied as expected by the loss of *in vivo* killing activity. The *in vitro* activity of the cloacin part of the complex is not affected by this modification, in contrast to that of colicin E3, which was found to be enhanced. Also in contrast to colicin E3 (21) chemical modification of the primary amino groups of cloacin affects both the endoribonucleolytic and the *in vivo* killing activity (22).

In conclusion, it can be said that these chemical modifications have shown that probably a tyrosine residue is involved in the enzymic activity of cloacin, that a few specific carboxyl groups are essential for the receptor binding activity, but that other, hydrophobic interactions, probably also play a role in both processes.

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