Communication

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 263, No. 20, Issue of July 15, pp. 9573-9575, 1988
© 1988 by The American Society for Biochemistry and Molecular Biology, Inc.
Printed in U.S.A.

Structure and Bactericidal Activity of an Antibiotic Dodecapeptide Purified from Bovine Neutrophils*

(Received for publication, February 16, 1988)

Domenico Romeo‡, Barbara Skerlavaj‡, Martino Bolognesi§, and Renato Gennaro‡

From the ‡Department of Biochemistry, Biophysics, and Macromolecular Chemistry, University of Trieste, 34127 Trieste, Italy and the §Department of Genetics and Microbiology, University of Pavia, 27100 Pavia, Italy

Cytoplasmic granules of neutrophils store a variety of cationic polypeptides, which exert *in vitro* a potent antibacterial action and are potentially involved in host defense mechanisms. From an acid extract of bovine neutrophil granules we have purified over 2000-fold a dodecapeptide exhibiting bactericidal activity against both *Escherichia coli* and *Staphylococcus aureus* at 10^{-7}–10^{-5} M concentration. The purification procedure involved only two steps of ion-exchange and reversed-phase chromatography. The peptide, named bactenecin, has the amino acid sequence, Arg-Leu-Cys-Arg-Ile-Val-Val-Ile-Arg-Val-Cys-Arg, maintained in a cyclic structure by a disulfide bond between the two cysteine residues. Computer modeling of the dodecapeptide resulted in a conformation in which the chain adopts an antiparallel extended structure forming a γ turn at residue 7.

Insects and vertebrates produce polypeptides which exert a direct toxic effect *in vitro* on bacteria and parasites, thereby presumably contributing to the defense mechanisms of the host against invading microorganisms (1–3). In particular, neutrophils, eosinophils, and macrophages, which constitute the major animal cell-dependent defense system, synthesize highly cationic polypeptides, which can cause a marked decrease in viability of a variety of organisms (2, 4–9). For some of these polypeptides a mode of action has been reported consisting of an increase in permeability of the bacterial membrane (4) or of an inhibition of bacterial DNA synthesis (8).

In the bovine neutrophil the antibacterial activity is associated with a population of large granules which are the predominant organelles in the cytoplasm of this cell type (10). In previous investigations we have extracted and purified cationic proteins from the total population of bovine neutrophil granules and shown that they have toxic effects on a variety of bacteria (7, 8). We have collectively called these cationic polypeptides bactenecins (derived from the latin words "bacterium" and "necare" (to kill)).

Here we describe the purification and the antibacterial properties of a small bactenecin, which is a cyclic dodecapeptide.

EXPERIMENTAL PROCEDURES

Granules of peripheral bovine neutrophils were extracted in 0.2 M sodium acetate, 5 mM EDTA, pH 4, as previously reported (7), and the extracted proteins were fractionated to yield an over 2000-fold purified antibacterial dodecapeptide. Briefly, the extract was dialyzed against distilled water acidified with a few drops of concentrated HCl (pH ~3) in a Spectrapore 6 tubing (nominal M_r cut-off of 1000; Spectrum) and then vacuum-dried. The lyophilized powder was dissolved in 10 mM Na_2HPO_4, 0.2 M NaCl, pH 7, and, upon clarification by centrifugation for 40 min at 30,000 × *g*, the protein solution was applied to a column (1.5 × 20 cm) of CM-cellulose (CM52, Whatman) equilibrated in 10 mM Na_2HPO_4, 0.2 M NaCl, pH 7. After extensively washing the column with the same buffer, elution of the bound proteins was first carried out with 300 ml of 0.3 M NaCl followed by 700 ml of 1 M NaCl (both in 10 mM Na_2HPO_4, pH 7). The latter high ionic strength solution eluted three peaks. The fractions of the last peak were combined into a pool, which was dialyzed under the conditions described above. After vacuum drying, the residue was solubilized in 0.1% trifluoroacetic acid and passed through a Pep-RPC HR 5/5 column of the Pharmacia LKB Biotechnology Inc. fast protein liquid chromatography system. Elution of the peptides, which was monitored at 214 nm, was performed with a 0–100% gradient of acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 0.7 ml/min. The resolved peaks were lyophilized after evaporation of acetonitrile by flushing with oxygen-free nitrogen.

Protein was determined by the method of Lowry *et al.* (11) with bovine serum albumin as standard. The concentration of the purified antibacterial peptide was also checked by amino acid analyses, assuming integral values for all the amino acid residues (see Table I). These analyses repeatedly yielded a correlation factor of 0.68 with respect to the values by Lowry's method.

Acid PAGE[1] (12) was carried out at 150 V on 16.5-cm slabs, and the peptides were stained with Coomassie Brilliant Blue or silver.

After hydrolyzing dried aliquots of the purified bactenecin (~10 nmol) for 22, 48, or 72 h at 110 °C in evacuated sealed tubes with 0.2–0.3 ml of 6 N HCl, their amino acid content was analyzed with an automatic amino acid analyzer (C. Erba, Milano, Italy).

For carboxymethylation, ~60 nmol of the purified bactenecin in 160 μl of 1 M Tris-HCl, pH 8.3 was reduced under nitrogen at room temperature for 2 h with a 10-fold molar excess of dithiothreitol. Alkylation of thiol groups was performed for 1 h in the dark with recrystallized iodoacetic acid in a 1.1-fold molar excess over the total –SH concentration. At the end of this procedure, the carboxymethylated peptide was separated from all the reagents by HPLC.

Amino acid sequencing of underivatized (~10 nmol) and carboxymethylated (~2.5 nmol) samples was carried out with a model 470A protein Sequencer (15–20 cycles, 94–96% repetitive yield), and the resultant phenylthiohydantoins from each cycle were analyzed on HPLC by the on-line model 120A phenylthiohydantoin analyzer (Applied Biosystems).

Titration of potentially free –SH groups was carried out with 5,5′-dithiobis-(2-nitrobenzoic acid) with reduced glutathione as standard (13).

Modeling of the dodecapeptide was performed on an Evans and Sutherland PS300 interactive graphics display by means of the program FRODO (14). An extended oligopeptide chain corresponding to the bactenecin sequence (see "Results and Discussion") was generated and subsequently folded to a cyclic structure connecting SG atoms of Cys-3 and Cys-11. The conformation of the oligopeptide loop was adjusted to attain proper stereochemistry, as judged from inspection of geometric stereochemical parameters and nonbonded interatomic

* This work was supported by grants from the Italian Ministry of Education and from the Italian National Research Council (Progetto finalizzato "Controllo delle malattie da infezione," Grant 86.01613.52). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; CFU, colony forming units; PBS, phosphate-buffered saline.

This is an Open Access article under the CC BY license.

contacts. The resulting atomic coordinates were subjected to 50 cycles of energy refinement (15).

Staphylococcus aureus ATCC 25923 and *Escherichia coli* ATCC 25922 were grown in Iso-Sensitest broth (Oxoid, UK), and the minimal inhibitory concentration of the bactenecin was assayed by a microdilution susceptibility test (8). The bactericidal activity was evaluated by counting on plates the colony forming units (CFU) of bacteria treated at 37 °C for 0.5 or 2 h with various concentrations of bactenecin in a medium preventing bacterial growth (PBS: 10 mM sodium phosphate, pH 7.2, 127 mM NaCl, 3.8 mM KCl, 0.5 mM Mg_2SO_4, 0.9 mM $CaCl_2$).

RESULTS AND DISCUSSION

Treatment of granules with 0.2 M sodium acetate, 5 mM EDTA, pH 4 extracted about 60% of the protein. Thus, granules of 5×10^{11} neutrophils, processed in several batches, produced 5.3 g of solubilized protein, which yielded 2.4 mg of a pure antibacterial dodecapeptide.

About 10% of the proteins solubilized from the granules were retained by CM-cellulose, equilibrated with 0.2 M NaCl, 10 mM sodium phosphate, pH 7. The pool of the most cationic peptides, emerging from the CM-cellulose column at the end of the elution with 1 M NaCl, 10 mM sodium phosphate, pH 7, was resolved into a limited number of peaks by hydrophobic reversed-phase chromatography (Fig. 1). The first peak contained a homogeneous peptide as revealed by both a second passage through the Pep-RPC column and by acid PAGE (see *inset* of Fig. 1; identical results, not shown, were obtained after staining the gel with silver stain) and exhibited a bacteriostatic activity with minimal inhibitory concentrations against *E. coli* and *S. aureus* of 8–25 and 1–8 μg/ml, respectively.

Table I shows the amino acid composition of the purified bactenecin. Automatic sequencing of the peptide confirmed its amino acid composition and yielded the following sequence: Arg-Leu-Cys-Arg-Ile-Val-Val-Ile-Arg-Val-Cys-Arg. The absence of free -SH groups in the dodecapeptide was also demonstrated by its failure to react with 5;5′-dithiobis-(2-nitrobenzoic acid).

The presence of a disulfide link was a key restraint in the modeling process since it required peptide chain reversal within 4 residues starting from Cys-3. In the conformation proposed (Fig. 2) the peptide chain adopts an antiparallel extended structure forming a bend containing three α carbon atoms (γ turn) at residue 7 (16). This kind of turn has been observed in thermolysin (17) and was predicted on theoretical grounds by Venkatachalam (18). In the model the disulfide connection is in the right-handed conformation, and the corresponding dihedral angles fall within those observed in globular proteins (19). The total conformation energy associated with the reported structure is -22.1 kcal mol^{-1} (15). Detailed inspection of the molecule, however, shows that, with a more regular disulfide conformation, additional stabilization could be achieved. This is not possible without major alterations of the polypeptide conformation. It is thus likely that the structure shown in Fig. 2 is only a local minimum and that different conformations could be attained by bactenecin in the presence of solvent and/or other stabilizing factors such as hydrogen bonding to a receptor site.

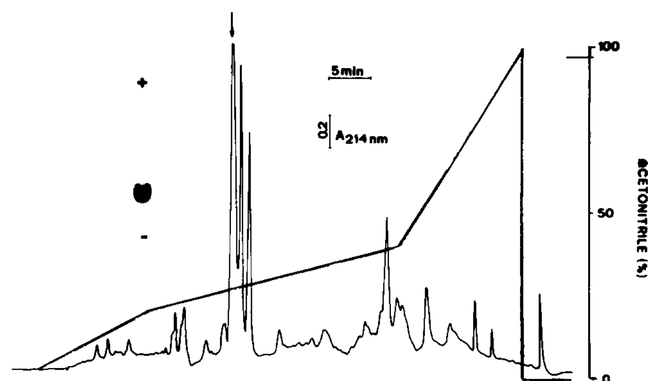

FIG. 1. **Reversed-phase chromatography of the peptide pool emerging from the CM-cellulose column at the end of elution with 1 M NaCl, 10 mM sodium phosphate, pH 7.** The graph shows a typical elution profile from the Pep-RPC 5/5 column eluted with a gradient of 0–100% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 0.7 ml min^{-1}. The *arrow* indicates the peak containing the bactenecin, and the *inset* shows an acid PAGE (Ref. 12) of this dodecapeptide (10 μg) after staining with Coomassie Brilliant Blue.

TABLE I
Amino acid composition of bactenecin

Samples (~10 nmol) of two different preparations of bactenecin were hydrolyzed at 110 °C in 6 N HCl (two samples from one preparation for 22 and 72 h, and one sample from the other preparation for 48 h). Data of $(Cys)_2$ also include the small amounts of Cys-SO_3^- generated during sample hydrolysis.

Amino acid	Amount in the peptide			Nearest integer
	22 h	48 h	72 h	
		mol/mol		
Val	2.20	2.56	2.76	3
$(Cys)_2$	1.10	0.94	0.88	1
Ile	1.11	1.58	1.92	2
Leu	1.09	1.02	1.00	1
Arg	3.79	3.70	3.88	4

The mechanism of action of low M_r bactenecin may encompass an interaction with and a translocation through the bacterial membrane(s). Actually, after 2 h of incubation it was equally effective as a bactericidal agent on *E. coli* and *S. aureus*, decreasing their viability by 50% at 1 μM concentration (Fig. 3), but with shorter incubations (30 min) the dodecapeptide was much less active on *E. coli* than on *S. aureus*. The surface organization in Gram-negative bacteria is considerably more complex than in Gram-positive cells with a lipopolysaccharide-containing outer envelope surrounding the peptidoglycan layer and the cytoplasmic membrane. Thus, an effective dose of bactenecin might require a longer time for passing through the envelope of *E. coli* than through the membrane of *S. aureus*.

We have found that about 5 ng of dodecapeptide can be extracted from 1×10^6 neutrophils (0.350 μl of cell volume). Assuming that the vacuoles of 1×10^6 actively phagocytizing neutrophils have a volume of about 0.05 μl and if only 10–20% of the granule contents is discharged into the vacuoles, the concentration reached by the dodecapeptide in the vacuoles would be 7–14 μM. From the experiments reported in Fig. 3, this concentration would be sufficient for virtually sterilizing the contents of the vacuoles.

We have recently found (20) that both low M_r (1,600–8,000) antibacterial peptides and high M_r (15,000–25,000) inactive polypeptides, all purified from granule extracts of bovine neutrophils, share immunoreactivity to a monoclonal antibody, BP97. This observation suggests that the granule-associated defense system of the neutrophil may arise from larger progenitor molecules by proteolytic cleavage. Processing of a protein precursor has also been reported for the 34 residue antibiotics nisin and subtilin, synthesized by *Streptococcus lactis* and by a particular strain of *Bacillus subtilis* (21), and for the magainins, bactericidal peptides recently found in *Xenopus* skin (3).

The bactenecin here described also reacts with monoclonal antibody BP97 and exhibits a spectrum of biological activity identical to that of the 1.6-kDa peptide purified from granule

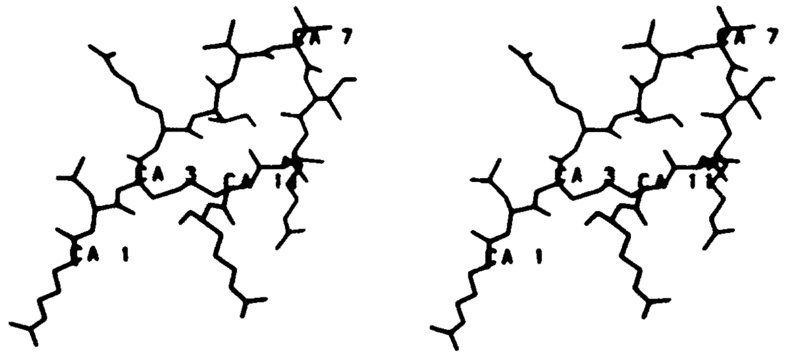

FIG. 2. **Bactenecin conformation.** The 3-11 disulfide bond is in the foreground, and the γ turn in the *upper right corner* (stereo view).

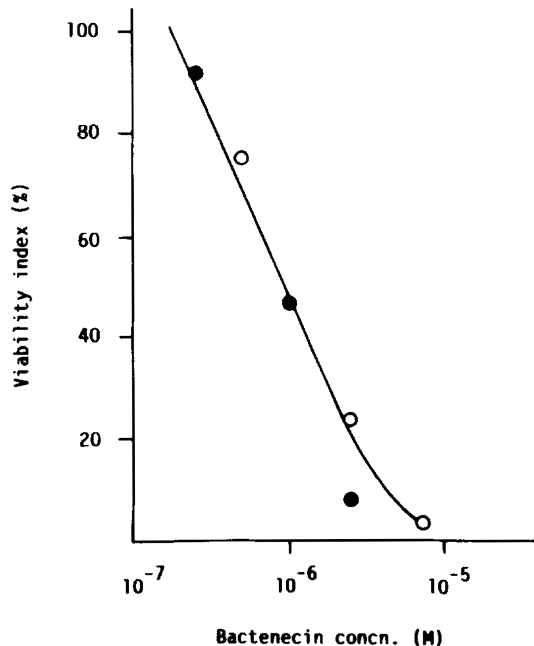

FIG. 3. **Bactericidal activity of the dodecapeptide bactenecin.** About 1 × 10^6 CFU of either *E. coli* (●) or *S. aureus* (○) from overnight cultures were incubated for 2 h at 37 °C in 0.2 ml of PBS in the absence or in the presence of various concentrations of bactenecin. The bacterial suspensions were then diluted severalfold in PBS and plated on nutrient agar. The viability index is the ratio $(CFU)_{treated}/(CFU)_{control}$ × 100.

extracts by immunoaffinity chromatography (20) with BP97 coupled to Affi-Gel 10. By extrapolation, we thus suggest that the present antibacterial dodecapeptide may be produced from a large inactive precursor by cleavage reactions carried out in the vicinity of an -S-S-bridge. This precursor would be processed into active antibacterial agents either *in vivo* when discharged into the phagocytic vacuoles together with granule proteases (10) or *in vitro* during granule extraction.

Acknowledgments—We would like to thank Dr. A. Fontana for guidance in the amino acid analysis, Dr. R. W. Blacher for determination of the amino acid sequence, and Dr. I. C. Gunsalus for helpful discussions.

REFERENCES

1. Boman, H. G., and Hultmark, D. (1981) *Trends Biochem. Sci.* **6,** 306–309
2. Spitznagel, J. K. (1980) in *The Reticuloendothelial System. A Comprehensive Treatise* (Sbarra, A. J., and Strauss, R. R., eds) Vol. 2, pp. 355–368, Plenum Publishing Corp., New York
3. Zasloff, M. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 5449–5453
4. Weiss, J., Elsbach, P., Olsson, I., and Odeberg, H. (1978) *J. Biol. Chem.* **253,** 2664–2672
5. Butterworth, A. E., Wassom, D. L., Gleich, G. J., Loegering, D. A., and David, J. R. (1979) *J. Immunol.* **122,** 221–229
6. Patterson-Delafield, J., Szklarek, D., Martinez, R. J., and Lehrer, R. I. (1981) *Infect. Immun.* **31,** 723–731
7. Gennaro, R., Dolzani, L., and Romeo, D. (1983) *Infect. Immun.* **40,** 684–690
8. Savoini, A., Marzari, R., Dolzani, L., Serranò, D., Graziosi, G., Gennaro, R., and Romeo, D. (1984) *Antimicrob. Agents Chemother.* **26,** 405–407
9. Ganz, T., Selsted, M. E., Szklarek, D., Harwig, S. S. L., Daher, K., Bainton, D., and Lehrer, R. I. (1985) *J. Clin. Invest.* **76,** 1427–1435
10. Gennaro, R., Dewald, B., Horisberger, U., Gubler, H. U., and Baggiolini, M. (1983) *J. Cell Biol.* **96,** 1651–1661
11. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275
12. Spiker, S. (1980) *Anal. Biochem.* **108,** 263–265
13. Ellman, G. L. (1959) *Arch. Biochem. Biophys.* **82,** 70–77
14. Jones, T. A. (1978) *J. Appl. Crystallogr.* **11,** 268–272
15. Jack, A., and Levitt, M. (1978) *Acta Crystallogr. Sect. A Cryst. Phys. Diffr. Theor. Gen. Crystallogr.* **34,** 931–935
16. Nèmethy, G., and Morton, P. P. (1972) *Macromolecules* **5,** 755–758
17. Matthews, B. W. (1972) *Macromolecules* **5,** 818–820
18. Venkatachalam, C. M. (1968) *Biopolymers* **6,** 1425–1436
19. Thornton, J. M. (1981) *J. Mol. Biol.* **151,** 261–287
20. Marzari, R., Scaggiante, B., Skerlavaj, B., Bittolo, M., Gennaro, R., and Romeo, D. (1988) *Infect. Immun.*, in press
21. Nishio, C., Komura, S., and Kurahashi, K. (1983) *Biochem. Biophys. Res. Commun.* **116,** 751–758