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Characterization of Bovine Neutrophil Antibacterial Polypeptides Which Bind to *Escherichia coli*

LAURA LITTERI AND DOMENICO ROMEO*

Department of Biochemistry, Biophysics, and Macromolecular Chemistry, University of Trieste, 34127 Trieste, Italy

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Bovine neutrophils contain several cationic polypeptides which exert potent microbicidal effects in vitro. To better characterize the repertoire of these polypeptides, we have incubated extracts of bovine neutrophils or neutrophil granules at pH 4 or 7 with either a smooth strain of *Escherichia coli* or a rough one. Only a few polypeptides interacted with the bacterial surface and were subsequently desorbed with 200 mM $MgCl_2$, as revealed by gel electrophoresis and analysis of Western blots (immunoblots) with appropriate antibodies. Two of the main proteins appearing in Coomassie blue-stained gels have molecular masses of 53 and 15 kDa and correspond to the heavy and light chains of myeloperoxidase. Another prevailing protein band with a molecular mass of 31 kDa was purified and shown to be 87% identical to human azurocidin/CAP37 in its 22-amino-acid N-terminal sequence. Proteins separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted to nitrocellulose did not react with an antiserum to human bactericidal/permeability-increasing protein. Conversely, immunoglobulin G against Bac7 or Bac5, two members of the antimicrobial proline- and arginine-rich polypeptide family, recognized in Western blots both the inactive precursor molecules, proBac7 and proBac5, and the mature polypeptides.

Neutrophils occupy a central role in host defense and kill microorganisms by releasing cytotoxic substances into the phagocytic vacuoles. These cytotoxins include reactive derivatives of oxygen, generated by a membrane oxidase (2), and cationic oligo- and polypeptides derived from cytoplasmic granules (7). Several of these peptides have been already purified to homogeneity, sequenced, and cloned (1, 3, 4, 9, 16, 18, 26, 27). Of the peptide antibiotic arsenal of bovine neutrophils (14), four antimicrobial peptides—a cyclic dodecapeptide (19), a tridecapeptide amide (5, 21), and two proline-rich polypeptides known as Bac5 and Bac7 (6, 8, 20, 24, 27, 28)—have been well characterized.

Here, we have exploited the technique of affinity binding to *Escherichia coli* described by Mannion et al. (13) to search for other potential antimicrobial peptides active on gram-negative bacteria. In addition, this approach has permitted us to investigate whether the precursors of Bac5 and Bac7, which are the stored, inactive forms of the two antibiotics (20, 28, 29), also bind to *E. coli*. We have obtained evidence that at an acidic pH *E. coli* binds myeloperoxidase, a 31-kDa protein very likely corresponding to azurocidin/CAP37 (1, 3, 16, 18, 23), and the precursors of Bac7 and Bac5. Binding of the latter polypeptides, as well as of Bac7 and Bac5, is increased at a neutral pH, a condition which largely prevents myeloperoxidase interaction with bacteria.

MATERIALS AND METHODS

Preparation of extracts. Neutrophils were purified from 6-liter batches of fresh bovine blood by differential centrifugation followed by hypotonic lysis of contaminating erythrocytes (17). For cytoplasmic granule preparation, after resuspension in 0.34 M sucrose cells were disrupted in a Dounce homogenizer and granules were sedimented from postnuclear supernatants at 10,000 × *g* for 10 min.

Extraction of neutrophils was carried out by suspending the cells in 0.2 M Na acetyl (Ac)-HAc, pH 4.0 (4×10^8/ml), lysing by sonication (three times [1 min each] at 3.5 A (Branson Sonic Power model S75), and then gently stirring for 2 h at 4°C. Extraction of granules was conducted by stirring a granule suspension in 0.2 M NaAc-HAc, pH 4.0, for 2 h at 4°C after a brief sonication (5 s, 3.5 A). In both cases, the insoluble material was sedimented at 30,000 × *g* for 20 min and the supernatant was dialyzed against either 10 mM NaAc-HAc, pH 4.0, or 10 mM Na_2HPO_4-NaH_2PO_4, pH 7.0.

Bacterial strains and growth conditions. *E. coli* ATCC 25922 was grown in Mueller-Hinton medium; the rough strain, *E. coli* J5 (13), was grown in minimal salts medium buffered with triethanolamine, pH 7.7 to 7.9; and a clinical isolate of *Streptococcus agalactiae* was grown in brain heart infusion medium. Binding experiments were performed with stationary-phase overnight cultures. For bactericidal assays, these cultures were transferred to fresh medium (diluted 1/50) and grown to mid-logarithmic phase at 37°C. Densities of bacterial suspensions were determined at 600 nm and converted to CFU per milliliter by appropriate standard curves.

Binding to and desorption from *E. coli*. This procedure followed the protocol described by Mannion et al. (13). In brief, *E. coli* cells were resuspended in dialyzed neutrophil or granule extract (5×10^8/ml of extract), incubated at 37°C for 10 min with gentle stirring, and sedimented at 1,000 × *g* for 15 min at room temperature. The cell pellet was washed twice with 1/2 volume of either 10 mM NaAc-HAc, pH 4.0, or 10 mM Na_2HPO_4/NaH_2PO_4, pH 7.0, and resuspended in either of the two buffers in the presence of 200 mM $MgCl_2$ (5×10^9 cells per ml). After incubating for 15 min at 37°C with shaking, the bacteria were sedimented as described above and the supernatant was collected for further analyses.

SDS-PAGE and Western blot (immunoblot). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 12% acrylamide slab gels (11) and bands

* Corresponding author.

were visualized with Coomassie blue. Before electrophoresis, samples were boiled for 5 min in sample buffer with 5% mercaptoethanol, 10% glycerol, and 2% SDS. Protein transfer to nitrocellulose (25) was performed with a 20-min blot in a semidry electrophoretic transfer cell (Trans-Blot SD; Bio-Rad Laboratories, Richmond, Calif.). Proteins were then fixed to the nitrocellulose membrane by incubating them for 30 min with 0.2% glutaraldehyde in buffered saline (pH 7.4) and then quenching for 15 min with 50 mM NH_4Cl. After exposure (2 h) to rabbit immunoglobulin G (IgG) against either Bac7 or Bac5 (28) or human myeloperoxidase (Dakopatts, Glostrup, Denmark), blots were incubated for 90 min with horseradish peroxidase-conjugated anti-rabbit IgG (Amersham International) and the color reaction was developed with 4-chloro-1-naphthol (Bio-Rad). For immunodetection of bactericidal/permeability-increasing protein (BPI), transferred proteins were reacted first with a goat antiserum to human BPI (a gift of J. Weiss) and then with horseradish peroxidase-conjugated anti-goat IgG.

Reverse-phase HPLC. On some occasions, proteins desorbed from bacteria were further purified by reverse-phase high-pressure liquid chromatography (HPLC) with a Pep-RPC HR 5/5 column of the fast protein liquid chromatography system (Pharmacia LKB Biotechnology Inc., Uppsala, Sweden). Elution of the column, monitored at 214 nm, was performed with a 0-to-100% acetonitrile gradient in 0.1% trifluoroacetic acid at a flow rate of 0.7 ml/min.

Protein determination and amino-terminal sequencing of proteins. Protein concentration was measured by the bicinchoninic acid protein assay reagent (Pierce, Rockford, Ill.). Amino-terminal sequencing was done by gas-phase Edman degradation using an Applied Biosystems 470A sequenator with a reverse-phase C_{18} column. In some instances, protein bands were transferred from 10% polyacrylamide gels to Biotrans polyvinylidene difluoride membranes (ICN Biomedicals, Costa Mesa, Calif.) as for Western blots and then eluted from the membrane for amino-terminal sequencing.

Microbicidal assays. The bactericidal activity of the 31-kDa protein was tested at 37°C with mid-logarithmic-phase *E. coli* ATCC 25922 or *S. agalactiae* (ca. 10^6 CFU/ml) in 10 mM sodium phosphate, pH 7.0. After incubating for 1 h, the bacterial suspension was diluted severalfold with buffered saline, pH 7.4, and plated on agar for CFU evaluation.

RESULTS

Strategies followed for binding of polypeptides to and desorption from *E. coli* were based on some variations in the experimental conditions. In essence, these variations consisted of (i) binding and desorption at either an acidic or a neutral pH, (ii) alternative use of extracts of either neutrophil lysates or isolated granules, and (iii) employment of either a smooth strain of *E. coli* or a rough one.

When neutrophil extracts, dialyzed against 10 mM NaAc-HAc, pH 4, are incubated with the smooth strain, *E. coli* ATCC 25922, and bound proteins are desorbed from bacteria with 200 mM $MgCl_2$ and then analyzed by SDS-PAGE, only a limited number of bands appear in Coomassie blue-stained gels (Fig. 1, lane b). The main proteins have molecular masses of 53, 42, 31, and 15 kDa. Identical results are obtained when either granule extracts replace the crude neutrophil extracts or the neutrophil extracts are incubated with the rough strain, *E. coli* J5. The 53- and 15-kDa bands react with anti-human myeloperoxidase IgG in Western blots (not shown). Furthermore, after transfer to the Biotrans polyvinylidene difluoride membrane and amino acid sequencing, the 53-kDa band shows an N-terminal sequence (12 amino acids analyzed) homologous to that of the heavy chain of human myeloperoxidase (10). Western blots do not react with anti-human BPI serum (data not shown). On the contrary, when reacted with anti-Bac7 IgG, they reveal a clear band of proBac7 (Fig. 2, lane c), which is the prevailing form of this antimicrobial polypeptide in neutrophil extracts prepared and maintained at pH 4 (28). With anti-Bac5 IgG, Western blots also show a very faint band of proBac5 (Fig. 2, lane a).

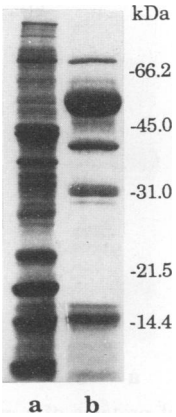

FIG. 1. SDS-PAGE of proteins of a neutrophil extract incubated with the smooth strain, *E. coli* ATCC 25922, in 10 mM NaAc-HAc, pH 4. Lane a, proteins not binding to bacteria; lane b, proteins bound to bacteria and desorbed with 200 mM $MgCl_2$.

If acid neutrophil extracts are brought to a neutral pH by dialysis against 10 mM Na_2HPO_4-NaH_2PO_4, pH 7.0, before incubation with *E. coli* ATCC 25922 in the same buffer, proteins desorbed from bacteria have, in general, masses lower than 31 kDa (Fig. 2, lane e). Under these conditions, Western blots of these proteins (Fig. 2, lanes b and d) reveal not only proBac5 and proBac7 but also the mature polypeptides Bac5 and Bac7, to which the precursors are converted in neutrophil or granule extracts at a neutral pH (28).

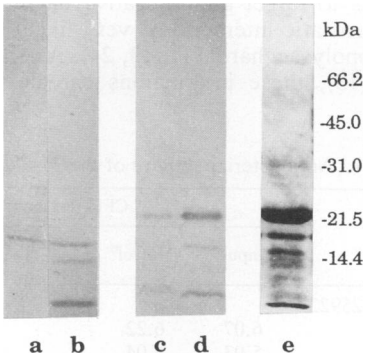

FIG. 2. SDS-PAGE and Western blot of neutrophil proteins bound to and desorbed from *E. coli* ATCC 25922. Lanes a and c, neutrophil extract in 10 mM NaAc-HAc, pH 4.0, and immunodetection with anti-Bac5 and anti-Bac7 IgG, respectively; lanes b and d, neutrophil extract in 10 mM Na_2HPO_4-NaH_2PO_4, pH 7.0, and immunodetection with anti-Bac5 and anti-Bac7 IgG, respectively; lane e, SDS-PAGE of proteins bound to and desorbed from bacteria in 10 mM Na_2HPO_4-NaH_2PO_4, pH 7.0, stained with Coomassie blue.

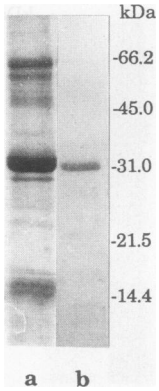

FIG. 3. SDS-PAGE of proteins of a granule extract in 10 mM NaAc-HAc, pH 4.0, bound to the rough strain, *E. coli* J5, and desorbed with 200 mM $MgCl_2$ (lane a) and of a 31-kDa protein purified therefrom by reverse-phase HPLC (lane b).

Another condition leading to a marked decrease of myeloperoxidase binding to bacteria is the reaction of granule extracts in 10 mM NaAc-HAc, pH 4, with the rough strain, *E. coli* J5. In this case, the main band seen in gels corresponds to the 31-kDa protein (Fig. 3, lane a). This protein can also be easily purified to homogeneity from the material desorbed from bacteria by reverse-phase HPLC (Fig. 3, lane b). Amino acid sequencing shows that the 31-kDa protein has an N-terminal sequence, IVGGRKARPQELPFLAS IQNQG, which is 87% identical to that of human azurocidin/CAP37 (1, 16, 18). The 31-kDa protein is bactericidal against *E. coli* and *S. agalactiae* at 20 μg/ml (Table 1), with a considerable antibacterial activity also observed at 2 μg/ml (not shown). Furthermore, comparison of the antimicrobial activity of this protein against *E. coli*, carried out in parallel in 10 mM Na phosphate, pH 7.0, and 50 mM Na citrate, pH 5.5 (3), reveals that the bactericidal effects are greater under acidic conditions (data not shown).

DISCUSSION

The initial binding of cationic antimicrobial polypeptides of neutrophils to target gram-negative bacteria appears to involve electrostatic interactions, very likely with the core region of lipopolysaccharide (1, 13, 24). As shown by Mannion et al. (13), these interactions can be exploited for affinity purification of the peptide antibiotics from crude extracts of neutrophils. Since a large portion of the bound proteins remains surface associated, these proteins can be displaced by high concentrations of $MgCl_2$ and then recognized by their bioactivity or reaction to specific antibodies or sequence analysis.

TABLE 1. Antibacterial activity of the 31-kDa protein[a]

Organism	CFU (log units)		
	Input	Control[b]	With the 31-kDa protein[b] (20 μg/ml)
E. coli ATCC 25922			
Expt 1	6.07	6.22	4.78
Expt 2	5.93	6.04	4.48
E. coli J5	5.98	6.12	5.19
S. agalactiae			
Expt 1	4.60	4.47	3.17
Expt 2	4.88	4.84	3.08

[a] Experiments were carried out at 37°C with mid-logarithmic-phase bacteria in 10 mM sodium phosphate, pH 7.0.
[b] 60-min incubation.

We have applied this technique to the characterization of some antimicrobial polypeptides present in extracts of bovine neutrophil lysates or neutrophil granules. We have found that under most experimental conditions a major protein binding to *E. coli* is myeloperoxidase. Electronmicroscopic examinations of phagocytosing neutrophils have revealed that myeloperoxidase is released into phagocytic vacuoles with a very rapid kinetics and much of the intraphagolysosomal enzyme is adsorbed to the surface of the ingested microorganisms (15, 29). In addition, several investigations carried out with purified myeloperoxidase and a variety of bacteria have shown extremely rapid and avid binding of the enzyme to the bacterial surface (15). Such binding has been suggested to be important in promoting the bactericidal activity of the myeloperoxidase-H_2O_2-Cl^- system (15, 22).

When using a rough strain of *E. coli* and crude extracts of human neutrophils, Mannion et al. (13) have found that there is a preferential binding of BPI to the bacterial surface and that large excesses of myeloperoxidase, lysozyme, or cathepsin G have little or no effect on BPI binding. By using an antiserum to human BPI, we have been unable to detect BPI in Western blots of bovine neutrophil proteins desorbed from either a smooth strain of *E. coli* or a rough one. A clone for bovine BPI has been isolated by screening a bovine bone marrow cDNA, and cDNA sequencing has indicated that there is a moderate (63%) conservation of amino acid sequences between human and bovine BPIs (12). The antiserum to human BPI might thus fail to recognize the bovine protein. Alternatively, BPI could be expressed in a small amount in bovine neutrophils, thus permitting extensive binding of other polypeptides to the *E. coli* surface under the experimental conditions employed.

Variation of experimental conditions, such as the use of extracts of isolated granules instead of neutrophil lysates and an acidic or a neutral pH during the binding procedure, has generated different binding patterns. These might be due to different solubilities of the cationic proteins in the extraction media. By these manipulations of the experimental conditions, we have been able to establish a preferential binding to *E. coli* of a 31-kDa polypeptide, which was subsequently purified by reverse-phase HPLC. This polypeptide has antimicrobial activity, a molecular mass, and an N-terminal amino acid sequence that are very similar to those of human azurocidin/CAP37 (1, 3, 16, 18, 23). Binding of the 31-kDa polypeptide to the *E. coli* surface membrane is consistent with previous suggestions (1, 23) that the antimicrobial activity of azurocidin/CAP37 might depend on an interaction with the anionic lipopolysaccharide.

We have previously shown that the bovine neutrophil antibiotics Bac5 and Bac7 are stored as precursors in the large granules (28, 29) and that these precursors acquire antimicrobial activity only after proteolytic processing by elastase (20). One possibility is that the precursors, proBac5 and proBac7, are inactive as antibacterial agents because they do not bind to the target bacteria. Experiments reported here have actually shown that both Bac5 and Bac7 and their precursors, present in crude extracts of neutrophils, bind to *E. coli*. Interaction with bacteria is much more evident when experimental conditions are set in such a way as to avoid

binding of other polypeptides such as myeloperoxidase and the 31-kDa protein. This is consistent with an obvious concept of competition of cationic antimicrobial peptides for anionic binding sites on the bacterial surface.

After binding to the envelope of gram-negative bacteria, Bac5 and Bac7 are likely to rapidly reach the inner membrane, where they generate a lesion, as indicated by unmasking of cytoplasmic β-galactosidase as well as by a fall in respiration-linked proton motive force (24). Thus, one possible explanation of the ineffectiveness as antibiotics of the large precursors of Bac5 and Bac7 might be found in their inabilities to gain access to the cytoplasmic membrane.

In conclusion, we have shown that the affinity purification technique of Mannion et al. (13), as slightly modified here, is suitable for screening crude extracts of bovine neutrophils for the presence of various cationic antimicrobial polypeptides as well as for gaining some insights into the complex picture of relationships among the various granule polypeptides which are involved in either the oxygen-dependent or oxygen-independent pathways of host defense.

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