Characterization of Antibacterial COOH-terminal Proenkephalin-A-derived Peptides (PEAP) in Infectious Fluids

IMPORTANCE OF ENKELYTN, THE ANTIBACTERIAL PEAP<sub>209–237</sub> SECRETED BY STIMULATED CHROMAFFIN CELLS

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Proenkephalin-A (PEA) and its derived peptides (PEAP) have been described in neural, neuroendocrine tissues and immune cells. The processing of PEA has been extensively studied in the adrenal medulla chromaffin cell showing that maturation starts with the removal of the carboxyl-terminal PEAP<sub>209–239</sub>. In 1995, our laboratory has shown that antibacterial activity is present within the intragranular chromaffin granule matrix and in the extracellular medium following exocytosis. More recently, we have identified an intragranular peptide, named enkelytin, corresponding to the bisphosphorylated PEAP<sub>209–237</sub> that inhibits the growth of Micrococcus luteus (Goumon, Y., Strub, J. M., Moniatte, M., Nullans, G., Poteur, L., Hubert, P., Van Dorsselaer, A., Aniis, D., and Metz-Boutigue, M. H. (1996) Eur. J. Biochem. 235, 516–525). As a continuation of this previous study, in order to characterize the biological function of antibacterial PEAP, we have here examined whether this COOH-terminal fragment is released from stimulated chromaffin cells and whether it could be detected in wound fluids and in polymorphonuclear secretions following cell stimulation. The antibacterial spectrum shows that enkelytin is active against several Gram-positive bacteria including Staphylococcus aureus, but it is unable to inhibit the Gram-negative bacteria growth. In order to relate the antibacterial activity of enkelytin with structural features, various synthetic enkelytin-derived peptides were tested. We also propose a computer model of synthetic PEAP<sub>209–237</sub> deduced from <sup>1</sup>H NMR analysis, in order to relate the antibacterial activity of enkelytin with the three-dimensional structure. Finally, we report the high phylogenetic conservation of the COOH-terminal PEAP, which implies some important biological function and we discuss the putative importance of enkelytin in the defensive processes.

Secretry granules from adrenal medullary chromaffin cells contain a complex mixture of low molecular mass constituents such as catecholamines, ascorbate, nucleotides, calcium, and several water-soluble peptides and proteins. These components are released into the circulation in response to splanchnic nerve stimulation. Since relatively large amounts of proenkephalin-A (PEA)<sup>1</sup> and chromogranin-derived peptides are found in adrenal medullary chromaffin granules, these organelles have proven to be an excellent model to study intragranular processing of these proteins. Recently, we have characterized the processing of bovine chromogranins A and B in chromaffin granules and in the extracellular medium following their release from stimulated cultured chromaffin cells (1, 2).

PEA, the precursor protein of Met- and Leu-enkephalin, as well as larger enkephalin-containing peptides, is highly conserved from Xenopus (3) to human (4). Originally, PEA mRNA was described to be present in various brain regions, most notably in the striatum (5) as well as in neuroendocrine tissues, the pituitary (6), and adrenal gland (6, 7). In addition to their expression in neural tissues, PEA and its derived peptides (PEAP) are expressed in a variety of immune cells, including ConA-stimulated CD4 T lymphocytes (8), CD4 thymocytes (9), B lymphocytes (10), as well as T cell lines, macrophages, and mast cells (11). In adult thymocytes and T lymphocytes clones, PEA mRNA is not expressed constitutively, but is detected following cell activation. After exogenous administration, enkephalins affect several immunologic functions, including antibody production (12), NK cell activity against tumors and viral infections (13), macrophage and polymorphonuclear leukocyte functions (14, 15), graft rejections (16), and mitogen-stimulated lymphocyte proliferation (17). Recently, it was shown that very low concentrations of PEA and Met-enkephalin differentially affect IgM and IgG production by B cells (18). Thus, enkephalins can enhance or inhibit particular immune functions (13, 19). Moreover, in several studies, bidirectional effects were reported: low concentrations of enkephalins enhance, whereas higher concentrations inhibit the same immune function. Thus, it is generally accepted that enkephalins act as modulators of immune reactions, although their physiological function in the immune system remains unclear. In addition to its expression in cells of the immune system, PEA mRNA is expressed in other tissues, such as comprising

<sup>1</sup>The abbreviations used are: PEA, proenkephalin-A; HPLC, high performance liquid chromatography; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; PEAP, proenkephalin-A-derived peptides; PMNs, polymorphonuclear neutrophils; PAGE, polyacrylamide gel electrophoresis.

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the reproductive system (20, 21), heart (22, 23), and in many developing tissues during gestation and the early postnatal period (24, 25). Hence, it has been postulated that PEP may play a role in cell or tissue growth and differentiation. Recently, it has been reported that endogenous enkephalins induced in thymocytes, modulate their own expression and function to inhibit the proliferation of activated thymocytes (26).

Natural processing of PEA has been extensively studied. Since 1982, it has been well established that several opioid peptides including Met-enkephalin and Leu-enkephalin in the ratio 4:1, two COOH-terminal extended variants, Met-enkephalin-Arg-Phe \(^7\) and the octapeptide Met-enkephalin-Arg-Gly-Leu \(^8\) are liberated by cleavage of the precursor at pairs of basic residues. In these studies, high concentrations of COOH- or NH\(_2\)-terminal extended variants of these peptides have been found in bovine adrenal medullary chromaffin cells (27, 28). More recently, the processing of PEA has been well examined in adrenal medulla chromaffin cells (29), as well as in stably transfected mouse anterior pituitary tumor (AtT-20) cells (30), showing that PEA maturation proceeds through an orderly series of steps. Similarly to other precursors, PEA maturation appears to start with the removal of the carboxyl-terminal series of steps. Similarly to other precursors, PEA maturation proceeds through an orderly process. These three phosphorylation sites are clustered to appear to start with the removal of the carboxyl-terminal

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Thus, antibacterial peptide PR-39, initially identified in pig intestine kills bacteria as a non-immune defense mechanism (39) and induces mammalian cells to express cell surface heparyn sulfate proteoglycans (40) which are involved in the wound repair process (41). Since PEP also affect cell and tissue growth (24, 25), we decided to analyze infectious fluids with respect to the antibacterial potency of these peptides.

In addition, various natural and synthetic enkelytin-derived peptides were prepared and tested to identify the structural features necessary for a potent antibacterial activity toward \(M. \) \(luteus\). In 1996, according to the Homolog method provided in Pro-Explore, we reported comparative predictions of secondary structure of enkelytin (38) and the homologous diazepam-binding inhibitor-derived peptide (42), suggesting an amphipathic helical structure for PEP243-237. Here, we generate a computer three-dimensional structure for the synthetic PEP209-237 on the basis of our \(^1\)H NMR study and discuss these structural features in relation to the antibacterial activity of enkelytin. Finally, the phylogenetic features of the highly conserved enkelytin are reported on the basis of the alignment of PEA198-219 (according to bovine sequence) from several species, and discussed in terms of enkelytin biological importance.

**EXPERIMENTAL PROCEDURES**

**Isolation of Peptides and Proteins Released from Stimulated Cultured Cells**—Chromaffin cells were isolated from fresh bovine adrenal glands and cultured as described previously (1). Cells were plated at a density of 10⁵ cells/50-mm in plastic Petri dishes. After 3 days in culture, the medium was removed and cells were washed four times with Locke’s solution (140 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO \(_4\), 2.5 mM CaCl \(_2\), 11 mM glucose, 0.5 mM ascorbic acid, 15 mM Hepes, p\(H\) 7.5) and subsequently stimulated for 10 min with Locke’s solution containing 10 μM nicotine. External medium was carefully collected, completed with trisfluoroacetic acid up to 0.1%. Extracellular medium was lyophilized and stored at −20°C.

**Isolation of Peptides and Proteins Released from Polymorphonuclear Neutrophils**—Human PMNs were prepared to 98% homogeneity, as described previously (43), from buffy coats of healthy donors of either sex, kindly provided by the Center de Transfusion Sanguine de Strasbourg (France). PMNs were suspended in a buffer solution containing 140 mM NaCl, 5 mM KCl, 1.1 mM CaCl \(_2\), 0.1 mM EGTA, and 10 mM Hepes, p\(H\) 7.3, at 5 × 10⁵ cells/ml per plate. Exocytosis of the content of the specific and primary granules of PMNs was initiated at room temperature by application of 2.3 mM LukS-PV and 0.6 mM LukF-PV, the two components of leukocidin from \(Staphylococcus aureus\) (44). The secretion was monitored by flow cytometry as described previously (45) and, when completed, PMNs were centrifuged (800 × g) for 10 min. The supernatant was recovered for further analysis.

**Isolation of Proteins from Periarthritis Abscess Fluids**—Fluid collected from natural bovine knee periarticular abscess was extracted with 1 M acetic acid (\(\nu/\nu\)). After centrifugation at 12,000 rpm during 15 min at 4°C, the supernatant was collected and the soluble material was successively filtered through Millex filters 0.45 μm and 0.22 μm and then loaded on a HPLC column.

**Purification of PEP by Reverse Phase HPLC**—PEP were isolated from cell secretion and abscess fluids using the Applied Biosystems HPLC system 140 B. Reverse phase HPLC were successively performed on Macherey-Nagel Nucleosil columns. In some experiments, a final purification was performed on a Brownlee C18 column (0.5 × 150 mm; particle size 5 μm and pore size 300 Å). Absorbance was monitored at 214 nm and the solvent system consisted of 0.1% (\(\nu/\nu\)) trifluoroacetic acid in water (solvent A) and 0.1% (\(\nu/\nu\)) trifluoroacetic acid in acetonitrile (solvent B). Each HPLC elution was performed using a flow rate and gradient as indicated or shown on chromatogram.

**Western Blot Analysis**—Extracts of biological fluids were separated by SDS-PAGE acrylamide containing 17% acrylamide (46). In order to detect immunologically reactive fragments, proteins were electrically transferred to nitrocellulose sheets (47). Electrophotographic blots were stained with Ponceau red. They were first soaked in 3% bovine serum albumin in 25 mM sodium phosphate containing 0.9% NaCl at pH 7.5 (NaCUP). Nitrocellulose sheets were quickly washed with NaCUP, and incubated 2 h at room temperature with anti-PEP224-237 antiserum diluted in...
NaClP, (1/1000). The second antibody was an anti-rabbit IgG conjugated with alkaline phosphatase (Bio-Rad). The nitrocellulose sheets were stained for enzyme activity in 100 mM NaCl, 50 mM MgCl₂, 100 mM Tris/HCl, pH 8.5, containing 0.4 mM nitro blue tetrazolium (Boehringer) and 0.38 mM 5-bromo-4-chloro-3-indolyl phosphate (Boehringer Mannheim). The color reaction of cross-phosphorylated forms of PEAP209–239 (including fractions c and d) was attributed to strong, medium, and weak NOE, respectively. Back-calculated NOE maps were used to check the consistency of the resulting three-dimensional models with the experimental spectra and resolve the initial ambiguous NOE assignments through several runs of structure calculations. The program Insight II ( Biosym ) was used to visualize the structures.

**RESULTS**

To further characterize the biological role of enkelytin, we examined whether enkelytin is co-released with catecholamines from stimulated chromaffin cells and whether it is present in biological fluids, particularly those involved in immune reactions. In order to analyze its biological activity, an antibacterial spectrum was realized with the natural peptide. Several natural and synthetic enkelytin-derived peptides were also tested to determine the structural features necessary for the antibacterial activity. Then, the activity of these peptides was related with the α-helical structure, obtained from recent 1H NMR data (89).

**Characterization of Antibacterial COOH-terminal PEAP in Material Released from Stimulated Cultured Bovine Chromaffin Cells**—The complex mixture of chromogranins and PEAP recovered in the secreted material was subjected to separation by HPLC on a reverse-phase C18 column (Fig. 1A). The different peaks were directly tested for their antibacterial activity against *M. luteus* (see “Experimental Procedures”) and sequenced. Several peaks containing antibacterial peptides were eluted from the column and active PEAP were detected in areas 1 and 2 (including fractions 2a to 2c), eluted with acetonitrile at 38 and 42%, respectively. After automatic Edman degradation of these different fractions, a unique NH₂-terminal sequence was located at position 209 of PEAP. This sequence (Fig. 1B) possesses three putative phosphorylation sites (Ser₂¹⁻¹⁵, Ser₂²¹¹ and Ser₂²³⁷) (34) and two oxidized residues (Met₂²² and Met₂³⁷). The peptide material present in these fractions completely inhibited *M. luteus* (strain A270) growth at a concentration of 0.2 μM, but was inactive against *E. coli* (strain D22) in a similar range concentration. To determine the molecular differences between PEAP present in fractions 1, 2a, 2b, and 2c, the sequencing analysis was completed by a detailed study using MALDI-TOF MS. The mass spectra analysis of the peptides present in peak 1 (Fig. 1C) indicated, by comparison with the calculated molecular mass of peptide B (3658 Da), the presence of a major fragment with a molecular mass of 3836 Da corresponding to the monooxidized bisphosphorylated form of PEAP₂₀⁹–₂₃₉ (peptide B). Three other peptides were also identified as different forms of PEAP₂₀⁹–₂₃₉. Thus, the molecular masses of 3438 and 3516 Da are attributed to the mono- and bis-phosphorylated forms of PEAP₂₀⁹–₂₃₉ (calculated molecular mass of 3355 Da), while the higher masses 3754 and 3931 Da correspond to the monooxidized bisphosphorylated and to the dioxygen-dioxygenated form of PEAP₂₀⁹–₂₃₉ (calculated molecular mass of 3555 Da), while the higher masses 3754 and 3931 Da correspond to the monooxidized bisphosphorylated and to the dioxydiphosphorylated form of PEAP₂₀⁹–₂₃₉ (calculated molecular mass of 3658 Da). The occurrence of oxidation products was explained by the presence of two methionine residues (Met₂²² and Met₂³⁷) in the peptide B sequence (Fig. 1B). The experimental mass values obtained for fractions 2a to 2c indicated the exclusive presence of non-oxidized mono- and bisphosphorylated forms of PEAP₂₀⁹–₂₃₉ (data not shown).

From these studies, we can conclude that natural bisphosphorylated forms of PEAP₂₀⁹–₂₃₉ and PEAP₂₀⁹–₂₃₇ named peptide B and enkelytin, respectively, are co-released with catecholamines and other neuropeptides following nicotine...
stimulation of cultured chromaffin cells. These two peptides possess a potent antibacterial activity against *M. luteus* growth. To further characterize the biological function of these antibacterial PEAP, several biological fluids from injured animals with infection and polymorphonuclear neutrophil secretions were examined.

**Isolation and Characterization of Antibacterial PEAP from Infectious Fluids**—Periarthritis abscess fluid was collected from cow knee, extracted by 1 M acetic acid as reported under “Experimental Procedures” and submitted to a Western blot analysis against anti-PEA224–237 (Fig. 2D, lane 3). Two bands were immunodetected with molecular mass of 20 and 4 kDa, respectively. The broad strongly immunoreactive band (20 kDa) indicated the presence of several molecular species of PEAP. Sequencing analysis of this material confirmed that several forms of PEAP209–237, B, sequence of PEAP209–239, C, analysis by MALDI-TOF MS of the secreted peptides present in fraction I. By comparison with the calculated molecular mass of PEAP209–237 (3355 Da), the experimental masses (3438 Da and 3516 Da) correspond to the mono- and bisphosphorylated forms of PEAP209–237. The two other detected masses (3754 and 3931 Da) were characterized by comparing with the calculated molecular mass of PEAP209–239 (3658 Da) and correspond, respectively, to the monooxidized monophosphorylated and dioxidized triphosphorylated forms of PEAP209–239.

In order to isolate enkelytin, the acid extract was subjected to a first HPLC on reverse-phase Macherey-Nagel Nucleosil 100–5C18-HD column (4 × 250 mm) (Fig. 2A). Different fractions were collected, tested in antibacterial assays against *M. luteus*, and immunoreactivity with anti-PEAP224–237 antiserum was screened by Western blot analysis. The immunoreactive fraction (Fig. 2A, a) displayed a potent antibacterial activity against *M. luteus* and sequencing indicated the presence of a complex mixture of several peptides. In order to isolate the shortest antibacterial COOH-terminal PEAP, two additional HPLC were performed. Peptidic material contained in this fraction was first separated (Fig. 2B) on a reverse-phase Macherey-Nagel Nucleosil 300–5C18 column (2 × 125 mm) and for complete purification of the antibacterial immunoreactive fraction b, a third chromatography was performed on a Macherey-Nagel Nucleosil 300–5C18 column (3 × 250 mm) (Fig. 2C). Fractions c2 and c3 were immunoreactive with anti-PEAP224–237 antiserum and after sequencing fraction c1, we detected the NH2-terminal sequence of defensin BDO1 (DFASXHTNNI; P46159) (54) and the dodecapeptide (RLXRIVVIRVXR; P2226) (55). MALDI-TOF analysis have confirmed the presence of these two antibacterial peptides (4273 and 1485 Da, respectively).
Fig. 2. Characterization of PEAP from cow knee peri-arthritis abscess fluid. A. HPLC elution profile on a Macherey-Nagel reverse-phase Nucleosil 300–5C18-HD column (4 × 250 mm) of peptidic material included in an acid extract of cow peri-arthritis abscess fluid. Absorbance was monitored at 214 nm and elution was performed at a flow rate of 700 μl/min, with a linear gradient as indicated in the right-hand scale. Antibacterial activity and immunoreactivity with anti-PEAP224–237 were detected in fraction a. B. HPLC elution profile on a Macherey-Nagel reverse-phase Nucleosil 300–5C18 column (2 × 125 mm) of peptidic material included in fraction a. Absorbance was monitored at 214 nm and elution was performed at a flow rate of 400 μl/min with a linear gradient as indicated in the right-hand scale. Antibacterial activity and immunoreactivity were detected in fraction b. C. HPLC elution profile on a Macherey-Nagel reverse-phase Nucleosil 300–5C18 column (3 × 250 mm) of peptidic material included in fraction c. Absorbance was monitored at 214 nm and elution was performed at a flow rate of 400 μl/min with a linear gradient as indicated in the right-hand scale. Antibacterial activity was detected in fraction c1, c2, and c3. and immunoreactivity in fractions c1 and c3. D. Western blot analysis (17% SDS-PAGE) with anti-PEAP224–237 lane a, molecular mass standard; lane b, intragranular chromatin soluble material; lane c, peptidic material included in an acid extract of cow peri-arthritis abscess fluid; lane d, HPLC fraction c3; lane e, peptidic material included in an acid extract of cow post-caesarean abscess fluid; lane f, peptidic material from induced rabbit abscesses (see “Experimental Procedures”); lane 7, secretions released from human PMNs.

Sequencing of immunoreactive fraction c2 indicated the NH2-terminal sequences of the defensin BDO2 (VRNHVTXRINRG-FXVPFR; P46146) (54), bacterecin-5 (RFRPPRIPRFRP; P19660) (56), histone H2B2 (PEPAKSKAPAP; homologous to H2B2 histones of different species) (58–60). In addition, MALDI-TOF analysis (Fig. 3) confirmed the presence of COOH-terminal PEAP with experimental masses of 3516 Da (the bisphosphorylated form of PEAP209–237), 3508 Da (PEAP209–238), 3523 Da (the monooxidized form of PEAP209–238), and 3805 Da (the dioxidized triphosphorylated form of PEAP209–238 with addition of a sodium ion). We also detected a molecular mass of 7027 Da corresponding to a dimeric form of enkelyn (3516 Da). In some experiments, a narrow-bore HPLC was performed on a Brownlee C18 column. Elution was performed at a flow rate of 5 μl/min using successively 15% B over 15 min and a gradient of 5% B to 80% B over 105 min. This additional chromatography confirms the previous HPLC profile and corroborates the presence of PEAP199–237/238 (data not shown).

In order to confirm the presence of antibacterial peptides derived from the COOH-terminal end of PEAP within wounds, we examined two other infectious fluids. The first liquid was drained from a post-operative (post-caesarean) abscess in the subcutaneous lining of a cow. Western blots analysis with
an anti-PEAP 224–237 antiserum (Fig. 2D, lane 5) indicated similar immunoreactivity to that obtained with the periarthritic abscess (Fig. 2D, lane 3). In a second experiment, a rabbit abscess induced by subcutaneous injection of complete Freund’s adjuvant was drained 10 days later. The material collected was treated as for bovine knee periarthritic abscess fluid and loaded on a HPLC reverse-phase C18 column. The different fractions were tested for antibacterial assays against M. luteus and submitted to Western blot immunodetection with anti-PEAP 224–237 antiserum, sequencing, and MALDI-TOF MS. In the immunodetected fractions (Fig. 2D, lane 6), we identified the NH2-terminal sequence of two rabbit defensins, NP1 (P01376) and NP2 (P01377) (61). The experimental mass values of 3892 and 3849 Da obtained for these peptides correspond to the theoretical molecular masses of defensins NP1 (3893 Da) and NP2 (3850 Da). In addition, since PEA sequences in several species are highly conserved (38), the rabbit PEA sequences and experimental molecular masses were compared with rat PEA (62, 63). The most likely candidates for these fragments are the bisphosphorylated form of PEAP206–237 and the monophosphorylated form of PEAP 209–237 with experimental molecular masses of 4453 and 3851 Da, respectively, instead of 4453 and 3853 Da for rat PEA.

In conclusion, the experiments described here reveal the presence of several peptides with antibacterial activity in fluids from infected wounds: defensins, bactenecins, dodecapeptide as expected, and natural PEA, such as several forms of PEAP 209–237, PEAP 208–238, and the bisphosphorylated form of PEAP 209–237 (enkelytin). Quantification of isolated enkelytin present at the inflammatory area could be obtained from sequencing and its concentration in (bovine periarthitis abscess fluid) was estimated to be from 0.5 to 1 μM. In this concentration range, the peptide is fully potent, indicating that enkelytin locally experts genuine antibacterial activity in specific fluids. In contrast, circulating enkelytin concentration is much less as it was hardly detectable in plasma (data not shown). As a continuation of this study, we have examined the presence of antibacterial PEA in secretions from human PMNs. After reverse phase HPLC on a Macherey-Nagel Nucleosil 100–5C18-HD column (4 × 250 mm), immunoreactivity was detected with anti-PEAP 224–237 antiserum (Fig. 2D, lane 5), indicating that PEA are secreted for PMNs with a pattern similar to those described for bovine periarthritic fluid (Fig. 2D, lane 3) and rabbit abscesses (Fig. 2D, lane 5).

Comparison of the Antibacterial Activities of Natural and Synthetic Enkelytin- and Peptide B-derivated Fragments—In order to further extend the bacterial spectrum initially reported for enkelytin (38), we decided to test the antibacterial activity of this natural peptide against several Gram-positive and negative bacteria. The data reported on Table I show that enkelytin entirely inhibits M. luteus and B. megaterium growth at 0.2 μM; it also inhibits the growth of S. aureus, being fully active at a concentration of 4.5 μM. Enkelytin was inactive toward B. subtilis under similar experimental conditions. Four different strains of E. coli (D22, D31, 663, and a wild strain, T13773) were tested with a peptide concentration of 3 μM but no antibacterial activity was detectable. These tested concentrations were in accordance with the amount of enkelytin found within physiological fluids. To conclude, this analysis spectrum indicates that the antibacterial activity of natural enkelytin is selective for several Gram-positive bacteria strains. In addition, it is important to point out that this new antibacterial peptide is able to inhibit the growth of S. aureus.

In order to characterize the structural features necessary for the antibacterial activity of enkelytin, we have tested several natural and synthetic PEAP against the growth of Gram-positive (M. luteus, strain A270) and Gram-negative (E. coli strain D22) bacteria. Natural enkelytin, PEAP 209–237 (peptide 1, Fig. 4A) and natural bisphosphorylated PEAP 209–239, known as peptide B (peptide 2, Fig. 4A) completely inhibit the growth of M. luteus at a concentration of 0.2 μM (Fig. 4B), but were unable to inhibit that of E. coli in the concentration range from 0.2 to 3 μM.

After preparation of synthetic enkelytin (bisphosphorylated PEAP 209–237), the peptide was loaded on a reverse phase chromatography. The HPLC profile and the MALDI-TOF MS indicated the presence of different molecular forms. Therefore, synthetic active enkelytin was further purified on a Macherey-Nagel Nucleosil 300–5C18 column (125 × 3 mm) and analyzed by sequencing and MALDI-TOF. After purification and sequencing of the active synthetic form, we evaluated that only 10% of the synthetic peptide adopts a conformation with the effective antibacterial activity (peptide 3, Fig. 4A). At this stage, its activity was closer to that of the natural peptide (100% of bacteria growth inhibition at 3 μM), in contrast with our previous work where we did not consider that only a low percentage of synthetic peptide adopts the active conformation.

<table>
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<tr>
<th>Bacteria</th>
<th>MIC (μM)</th>
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<tr>
<td>Gram-positive bacteria</td>
<td></td>
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<tr>
<td>M. luteus</td>
<td>0.2</td>
</tr>
<tr>
<td>B. megaterium</td>
<td>0.2</td>
</tr>
<tr>
<td>B. subtilis</td>
<td>&gt;3</td>
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<tr>
<td>S. aureus</td>
<td>4.5</td>
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<tr>
<td>Gram-negative bacteria</td>
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<tr>
<td>E. coli D22</td>
<td>&gt;3</td>
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<tr>
<td>E. coli D31</td>
<td>&gt;3</td>
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<tr>
<td>E. coli 663</td>
<td>&gt;3</td>
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<tr>
<td>E. coli T13773</td>
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Growth was assessed by measuring the increase at 37 °C with *M. luteus* (strain A 270) in yeast extract-free Luria-Bertani medium as described under “Experimental Procedures.” Microbial antibacterial assay of the NH2- and COOH-terminal domains (peptides 5 and 6) at a concentration of 500 µM showed neither any detectable antibacterial activity against this Gram-negative bacterium nor any hemolytic activity. In conclusion, the antibacterial activity of enkelytin toward *M. luteus* is directly related to three structural parameters: (i) the length of the peptidic chain, (ii) the natural conformational constraints induced by the three proline residues Pro212, Pro214, Pro227, and (iii) the phosphorylation of Ser221 and Ser223.

The presence of helical structure was confirmed in the 1H NMR spectra of synthetic PEAP209–237 by the presence of regular Hα(i), HN(i+3) NOE for residues from Ser221 to Gly230 and from Glu228 to Phe236. PEAP209–237 sequence contains three proline residues which are able to adopt either the cis or trans conformation of the peptide bond. The two isomers are characterized by distinctive NOE patterns between the protons of the proline and those of the preceding residue. Thus, each three proline residues showed different behavior: (i) Pro212 has a trans conformation, (ii) cis and trans NOE patterns were found for Pro227, but no different chemical shifts were observed for the two isomers. Therefore, two models were calculated with Pro227 either in the cis or trans conformation.

In both models (Pro227 cis and Pro227 trans) presented as a ribbon diagram (Fig. 5, A and B, respectively), the conformations of Pro212 and Pro214 were set to be trans. The Pro227

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**Fig. 4.** Antibacterial activity of natural and synthetic enkelytin-derived peptides. A, identification of the 8 different peptides tested. B, peptides at different concentrations were incubated 16 h at 37 °C with *M. luteus* (strain A 270) in yeast extract-free Luria-Bertani medium as described under “Experimental Procedures.” Microbial growth was assessed by measuring the increase at *A*$_{620}$nm. Values found with control cultures grown in the absence of peptide were taken as 0%. Numbers in each column indicate the peptide concentration inhibiting bacterial growth. Experimental values are given ± 5%.

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**Fig. 5.** Three-dimensional structure of enkelytin corresponding to PEAP209–237. Ribbon representation of the three-dimensional structure of PEAP209–237, in a 50% trifluoroethanol/water solution according to X-PLOR program (53). Both cis (A) and trans (B) conformations of Pro227 are deduced from the 1H NMR data (89). The NH$_2$-terminal parts of the two models (Phe227 to Pro227) have the same orientation. E, glutamic acid residue; P, proline residue; S, serine residue.
residue induces a bend in the three-dimensional structure, which adopts a L shape and breaks the helical structure observed on either side of Pro227. It is striking that both isomers of Pro227 lead to the same kind of spatial proximity between a glutamic acid and a serine side chain (Ser223/Glu230, in the cis conformation and Ser221/Glu228 in the trans one). In enkelytin, when the two serine residues (Ser221 and Ser223) are phosphorylated, the negatively charged phosphate groups probably induce conformational change by electrostatic interactions (64). In contrast to the COOH-terminal fragment 227–237 which adopts a helical conformation, the structure of the NH2-terminal end (fragment 209–214) is poorly defined, due to the lack of medium range NOE, partially explained by an averaging over a broad range of conformations resulting in the cis-trans isomerism of Pro214.

DISCUSSION

Despite intensive research to counter the development of new bacterial resistance, no novel classes of antibacterial agents have been discovered in the past 30 years. Currently, there is a great interest in antibacterial peptides as an attempt to resolve this challenge. Thousands of such molecules have been synthesized, but only a few, such as magainins, are commercially available in clinical trials. Thus, the structural and biological characterization of new antibacterial peptides, derived from naturally processed precursors is a topic of intense research. The antibacterial activity of enkelytin as described previously for a number of enkephalin analogs (69, 70) and the conformational state of the NH2-terminal Met-enkephalin as we report here (Fig. 5), is important to note that Pro227, which is highly conserved in PEA sequence from several species (Fig. 6), is breaking a regular helical conformation with a bend formation. This bending brings the glutamic acid residues (Glu228 and Glu230) close to the phosphorylated serine residues (Ser221 and Ser223). The repulsive electrostatic interactions resulting from the phosphorylation of Ser221/223 may act as molecular switch for the antibacterial activity. Thus, the phosphorylation of Ser221 and Ser223 by addition of negative charges could open the “boomerang angle” (38) or increase the ability of this peptide to bind divalent ions and thus induce the antimicrobial activity of enkelytin as described previously for a poly(Asp) antibacterial peptide (67, 68). The confirmation of this model will be provided by 1H NMR studies of the bisphosphorylated synthetic PEA209–237 and the bisubstituted glutamic acid at the phosphorylated serine residues (Ser221 and Ser223). The repulsive electrostatic interactions resulting from the phosphorylation of Ser221/223 may act as molecular switch for the antibacterial activity. Thus, the phosphorylation of Ser221 and Ser223 by addition of negative charges could open the “boomerang angle” (38) or increase the ability of this peptide to bind divalent ions and thus induce the antimicrobial activity of enkelytin as described previously for a poly(Asp) antibacterial peptide (67, 68). The confirmation of this model will be provided by 1H NMR studies of the bisphosphorylated synthetic PEA209–237 and the bisubstituted glutamic acid at the phosphorylated serine residues, in aqueous solution including divalent ions and in membrane environment. Moreover, it is interesting to point out that the helical structure for the COOH-terminal Met-enkephalin as we report here differs significantly from 1H NMR structures previously described for Met- or Leu-enkephalin (69, 70). This is probably due to the extension of the NH2-terminal region.

Antibacterial peptides have to be positively charged in order to bind to bacterial surfaces, which are usually negatively charged. Curiously, the net charges of the most active peptides numbered 1 to 3 (Fig. 4), were calculated to be −7, −6, and −7, respectively. However, enkelytin and peptide B, although negatively charged, may act by a pore-forming or carpet-like mechanism, as recently described (71). However, other mechanisms can also be considered such as peptide membrane receptors on
bacterial membranes, the possibility for the peptides to act as “oblique-oriental” peptides (72) or the ability for these anionic peptides to bind divalent ions (67, 68). At this stage, however, the mechanism by which enkelytin and peptide B inhibits bacteria growth remains to be determined. The presence in infectious fluids of antibacterial COOH-terminal PEAP together with other antibacterial peptides supports their potential role in host defense. Defensins and bactenecins are thought to be released at infection and inflammation sites. In the present study, several purification steps were necessary (3 successive HPLC) to isolate the different forms of active PEAP from periarthritis abscess fluid, suggesting that interactions occur between these acidic fragments and the cationic antibacterial peptides, such as defensins or bactenecins. The formation of molecular complexes including several peptides may be important to obtain a synergistic antibacterial efficiency. The computer model obtained for the synthetic PEAP209–237 (Fig. 5) indicated a long amphipathic α-helical structure. This structure completes our previous predicted model concerning the α-helical structure of PEAP224–237 (38).

PEAP209–239 is the most highly conserved domain of the protein precursor with a yield of homology around 90% (Fig. 6). Proline residue located in position 212 in bovine sequence is changed to Ala, Ser, Glu, or Phe residues in other species. Because of the high conservation of the COOH-terminal domain of PEA, the antibacterial activity appears to have occurred early in evolution.

The antibacterial COOH-terminal PEAP may originate from chroammofins, since these cells contain high levels of PEAP, or from immune cells (e.g., PMNs). PEA has been reported to be significantly expressed in the immune system and may provide a basis for neuroimmune interactions (8–11). The local inflammatory response initiates the synthesis and the secretion of opioid peptides by immune cells. When Freund’s adjuvant is used to induce unilateral hindpaw inflammation in rats, PEA mRNA are abundant in cells of the inflamed tissue, but absent in non-inflamed tissue. Numerous cells infiltrating the inflamed subcutaneous tissue are stained intensively with Met-enkelytin, suggesting that PEAP are synthesized and processed within various types of immune cells at the site of inflammation (73). Moreover, exposure of rats to lipopolysaccharide endotoxin leads to PEA mRNA and protein expression in macrophages within lymph nodes and in chroammofin cells within adrenal glands (74). One physiological effect of PEAP is to up-regulate or enhance the immune response at low concentrations, but this effect is abolished at high concentrations.

Other studies performed in invertebrates suggest a potential dual role of PEA in defensive processes (75, 76). Thus, enkelytin degradation at the infection site by two endopeptidases, neuropeptide-degrading endopeptidase and angiotensin-converting enzyme present in granulocytes, generate Met-enkelytin and its derived peptides (76). Met-enkelytin enhances the immune reaction in patients with cancer or AIDS (77). With regard to this immune modulating property, Met-enkelytin has been proposed to be classified as a cytokine (78). Moreover, this pentapeptide can bind opioid receptors present in peripheral inflamed tissues to mediate an analgesic effect (79). The involvement of opioids in neuroimmunoregulatory events appears to have a long evolutionary history. Although the relationship between the immune and nervous systems was discovered in vertebrates, it also exists in invertebrates (80) and the co-release of enkelytin and Met-enkelytin represents an unified neuroimmune protective response to stress situations that may be accompanied with infectious diseases. Taken together, these two peptides would provide a highly beneficial survival strategy at the very beginning of a proinflammatory process.

Our studies provide new data concerning the biological characterization of the COOH-terminal antibacterial PEAP named enkelytin, first isolated from chromaffin granules and now recovered as secretory products from stimulated chroammofin cells and in wound fluids. In view of the widespread distribution of PEA, these peptides may also be present and secreted from other endocrine, neuroendocrine, and immune cells. Due to their nonspecific action on membranes, the antibacterial peptides possess cytotoxic activities and may not only play a role in antimicrobial defense, but also in inflammatory processes.

Since antibacterial PEAP are released with catecholamines and chromogranins, the latter being precursors to other peptides with antibacterial activities (87), they may play a role in stress situations and act as one immediate protective barrier against infection. The identification of different classes of antibacterial peptides in a diverse range of organisms, including prokaryotes, insects, frogs, and mammals, suggests that they play a potentially important role in host defense against microbial infections.

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REFERENCES

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