Structure–activity analysis of thanatin, a 21-residue inducible insect defense peptide with sequence homology to frog skin antimicrobial peptides

Pascale Fehlbaum*†, Philippe Bulet*‡, Serguey Chernysh§, Jean-Paul Briand§, Jean-Pierre Roussel*, Lucienne Letellier†, Charles Hetru* and Jules A. Hoffmann*

*Réponse Immunitaire et Développement chez les Insectes, Unité Propre de Recherche 9022, and ‡Immunologie des Peptides et Virus, Unité Propre de Recherche 9021, Centre National de la Recherche Scientifique, Institut de Biologie Moléculaire et Cellulaire, 15, rue René Descartes, 67084 Strasbourg Cedex, France; §Laboratory of Entomology, St. Petersburg State University, Gromienbaumskoye ul., 2, 198904 St. Petersburg, Russia; and Laboratoire des Biomembranes, Unité de Recherche Associée 1116, Centre National de la Recherche Scientifique, Université Paris Sud, 91405 Orsay Cedex, France

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ABSTRACT Immune challenge to the insect Podisus maculiventris induces synthesis of a 21-residue peptide with sequence homology to frog skin antimicrobial peptides of the brevinin family. The insect and frog peptides have in common a C-terminally located disulfide bridge delineating a cationic loop. The peptide is bactericidal and fungicidal, exhibiting the largest antimicrobial spectrum observed so far for an insect defense peptide. An all-d-enantiomer is nearly inactive against Gram-negative bacteria and some Gram-positive strains but is fully active against fungi and other Gram-positive bacteria, suggesting that more than one mechanism accounts for the antimicrobial activity of this peptide. Studies with truncated synthetic isoforms underline the role of the C-terminal loop and flanking residues for the activity of this molecule for which we propose the name thanatin.

Three facets concur to the remarkably efficient host defense of insects: (i) cellular reactions; (ii) proteolytic cascades leading to localized coagulation and to melanin formation; (iii) synthesis in the fat body of potent antimicrobial peptides, which are secreted into the hemolymph to kill the invading microorganisms (review in ref. 1). Some 50 inducible antimicrobial peptides have been characterized from various insect species (2) and are conveniently grouped into four families: (i) the cecropins; (ii) the insect defensins; (iii) small (2–3 kDa) proline-rich; and (iv) large (10–30 kDa) glycine-rich peptides/polypeptides. In addition to antibacterial molecules, insects also produce inducible antifungal molecules (3).

We report here the isolation of an inducible defense peptide from the hemipteran insect Podisus maculiventris, which does not belong to any of the above-defined groups. Interestingly, it is the first insect defense peptide to show at physiological concentrations activity against Gram-positive and Gram-negative bacteria as well as against fungi. The novel peptide exhibits sequence similarity with the brevinin family of antimicrobial peptides from frog skin (4–8). We have synthesized an all-D-isof orm of the insect peptide, which is less active than the native peptide against Gram-negative and some Gram-positive bacteria. In contrast, it is as active against other Gram-positive strains and against fungi, suggesting that the peptide can affect microorganisms by more than one mechanism. We have synthesized several truncated isoforms and shown that a minimum motif consisting of the C-terminal loop and flanking residues can confer a detectable activity against some bacterial strains. We propose the name thanatin [from thanatos (death)] for this novel inducible insect defense peptide.

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Materials and Methods

Insects. Three-day-old P. maculiventris adults were pricked with a needle dipped into a pellet of heat-killed Escherichia coli and their hemolymph was collected after 24 h in the presence of aprotinin.

Purification of the Antibacterial, Antifungal Peptides. The cell free hemolymph was acidified with 0.05% trifluoroacetic acid and loaded on a Sep-Pak C18 cartridge (Waters), and stepwise elutions were performed with increasing proportions of 40% acetonitrile (20%, 50%, 80%). The fraction eluting at 50% acetonitrile was applied on serially linked size-exclusion columns (SEC 3000 and SEC 2000; 300 × 7.5 mm; Beckman) and eluted with 30% acetonitrile (flow rate, 0.5 ml/min). UV absorbance was monitored at 225 nm and antimicrobial activities were routinely monitored. The active fractions were applied on an Aquapure OD 300 C8 column (220 × 4.6 mm; Brownlee Lab) developed with a linear gradient of 2–52% acetonitrile over 90 min (flow rate, 1 ml/min). The antimicrobial molecule was purified to homogeneity on the same column with an appropriate biphasic gradient. See also refs. 9 and 10 for further details.

Peptide Synthesis. Fluoren-9-ylmethoxycarbonyl (Fmoc) amino acid derivatives (1 and 2-amino acids) were purchased from Neoysystem (Strasbourg, France). Assembly of the protected peptide chain was carried out on a 25 µM scale with a homemade multichannel peptide synthesizer according to classic Fmoc methodology as described (11). Cleavage from the resin and deprotection of the peptide were performed with reagent K (12) for 2 h. The peptide was collected in a tube filled with cold tert-buty1 methyl ether and after centrifugation the pellet was washed twice in cold ether and dissolved in water. Water and traces of reagents were removed by lyophilization. The peptide in reduced form was taken up in oxidation buffer (1 mg/10 ml) [0.1 M ammonium acetate (pH 8.5)] and was allowed to refold for 1 day at room temperature under stirring and was purified by semipreparative reversed-phase chromatography. Purity, sequence, and renaturation of the different isoforms of the peptide were confirmed by capillary electrophoresis, Edman degradation, and mass spectrometry. Peptide-free acids were assembled on a p-benzoxoxybenzyl alcohol resin and peptide amide was synthesized on a 4-[(2’,4’,6’-O-methoxyphényl)-Fmoc-aminomethyl]phenoxyacetamidoethoxy resin.

Assays. The strains were those used in previous studies (3, 10) plus Aspergillus fumigatus and Trichophyton mentagrophytes. Antimicrobial activities were monitored by liquid growth inhibition assays (3, 15).

Abbreviations: Fmoc, fluoren-9-ylmethoxycarbonyl, MIC, minimal inhibitory concentration.
†P.F. and P.B. contributed equally to this paper.
‡To whom reprint requests should be addressed.
Control antibiotic peptides MSI-94 (a broad-spectrum linear amphipathic magainin; ref. 14) and PGLa (a naturally occurring antibiotic from the frog; ref. 14) were a gift of M. A. Zasloff (Magainin Scientific Institute, Philadelphia).

Hemolytic activity was assayed spectrometrically on porcine red blood cells (3).

Agglutination assays were performed by incubating thanatin in 500 μl of Heps buffer (10 mM Heps/150 mM NaCl, pH 7.4/1 mM KCl/0.2% glucose) in the presence of a midlogarithmic-phase culture of E. coli 1106 (A600 = 0.5). For bactericidal assays, thanatin was incubated at 10 μM in 200 μl of a midlogarithmic-phase culture of E. coli D31 and 1106 at starting A600 = 0.001. Aliquots were removed at different time intervals and plated on nutrient agar. The number of colony-forming units was determined after an overnight culture at 37°C.

Microsequence Analysis. Automated Edman degradation was performed on a model 473A sequenator (Applied Biosystems).

Mass Spectrometry. The peptide was analyzed by electrospray ionization mass spectrometry on a VG BioTech BioQ mass spectrometer (Manchester, U.K.) as described (13).

RESULTS

Purification and Characterization of Thanatin. Hemolymph of untreated P. maculiventris adults is devoid of antibacterial activity, whereas a potent activity, directed against both E. coli and Micrococcus luteus, is present 24 h after bacterial challenge (data not shown). From the cell-free hemolymph of 250 challenged adults (3 ml), we have purified to homogeneity 15 μg of an antibacterial peptide also active against fungi. The peptide was sequenced by Edman degradation and consists of 21 residues, including two cysteines, the identity of which was ascertained by pyridylethylation (Fig. 1). The molecular mass of the peptide (2436.2 ± 0.22 Da) was in good agreement with the calculated mass (2436.2 Da), assuming that the two cysteines are engaged in an intramolecular disulfide bridge. The peptide is strongly cationic (pI 10.48) and resistant to pH variations (pH 2–10). The molecule is remarkable by its 6-residue strongly basic loop formed by the disulfide bridge binding amino acid 11 to residue 18. It shows no homology with other antibacterial peptides isolated from insects and was named thanatin. However, marked sequence similarity is evident with the brevinins, a family of antimicrobial peptides recently isolated from the skin secretions of various frog species (4–8) (Fig. 2).

Chemical Synthesis of Thanatin. To further investigate its properties, thanatin was synthesized by the Fmoc method. After renaturation, the sequence of the synthetic refolded compound was confirmed by Edman degradation, capillary electrophoresis, and mass spectrometry. Native and synthetic thanatin were active against E. coli 1106 and M. luteus at the same concentrations (see Table 1). The synthetic peptide was used for all experiments described below.

Activity Spectrum of Thanatin Against Bacteria (Table 1). Thanatin had a marked activity [minimal inhibitory concentration (MIC) < 1.2 μM] against the following Gram-negative bacteria: Salmonella typhimurium, E. coli, and Klebsella pneumoniae. It was moderately active against Erwinia carotovora (MIC 10–20 μM) and Pseudomonas aeruginosa (MIC 20–40 μM). Among the Gram-positive bacteria that were tested, Aerococcus viridans, M. luteus, Bacillus megaterium, and Bacillus subtilis were the most sensitive (MIC < 5 μM), whereas Pediococcus acidolactici was poorly sensitive (MIC 20–40 μM) and Staphylococcus aureus was not affected.

Thanatin appeared significantly more active than the standard antibacterial peptides MSI-94 and PGLa against E. coli, S. typhimurium, and K. pneumoniae but less active against A. carotovora and P. aeruginosa. In general, the activity of thanatin against Gram-positive cells was lower than that of these standards, with the exception of A. viridans.

Antifungal Activity of Thanatin (Table 1). Thanatin was also found to be active against fungi. The level of activity was high (MIC < 5 μM) against Neurospora crassa, Botrytis cinerea, Nectria haematococca, Trichoderma viride, Alternaria brassicola, and Fusarium culmorum. It was somewhat lower against Ascochyta pisi and Fusarium oxysporum (MIC < 20 μM). Thanatin was also active against two pathogenic fungi of humans, A. fumigatus and T. mentagrophytes (MIC 10–20 and 20–40 μM). Saccharomyces cerevisiae was not affected by concentrations up to 40 μM.

Testing of Hemolytic Activity. In a conventional assay on porcine erythrocytes, thanatin did not exhibit hemolytic activity even at 40 μM.

Mode of Action of Thanatin. Incubation of 10 μM thanatin for 4 h induced loss of viability of most bacteria of the E. coli D31 and 1106 strains. No bacteria survived after 24 h of exposure to thanatin (data not shown). Similar results were obtained for the other sensitive bacterial strains.

The primary effect of thanatin was the arrest, within the first 24 h of its addition, of the motility of E. coli cells. This was rapidly followed by agglutination into large clumps. Agglutination, as measured by the decrease of light diffusion at 600 nm, occurred with E. coli 1106, D31, and M. luteus but was observed only above a threshold concentration of thanatin dependent on the bacterial strain (8 μM for E. coli and 20 μM for M. luteus) and the ionic strength (2.5 μM in 10 mM NaCl and 8 μM in 150 mM NaCl) and could be reversed by addition of 1 mM CaCl2 (data not shown). To gain further understanding of the mechanism of action of thanatin, we checked for its effect on cytoplasmic membrane functions. E. coli D31 was incubated in Heps buffer at 37°C and its potassium content (K+), determined by using a K+ selective electrode (16). The presence of thanatin up to 70 μM did not induce any decrease of K+ as no variation of the K+ concentration (0.5 mM) could be detected in the buffer even 30 min after its addition, suggesting that the peptide had not permeabilized the inner membrane toward this cation. A similar result was obtained with M. luteus. Thanatin also did not increase the permeability of the inner membrane of M. luteus toward protons since the transmembrane electrical potential, determined from the accumulation of [3H]TPP+ (tetraphenylphosphonium ion), was unchanged (143 mV) with and without the peptide. The effect of thanatin on respiratory activity was measured by determining with a Clark electrode the oxygen consumption of E. coli and M. luteus cells in Heps buffer. The level of respiration remained at values around 35 nmol per mg of cells per min during the first hour of incubation in the presence of 40 μM thanatin regardless of whether the cells were aggregated or not (i.e., in the absence or presence of 1 mM CaCl2). Respiration started to decrease after 1 h and became undetectable at 6 h. This result corroborates the data on the bactericidal effect.

Table 1. Antimicrobial activity spectrum of thanatin, all-d-thanatin, C-amidated thanatin, and truncated isoforms

<table>
<thead>
<tr>
<th>Gram-positive bacteria</th>
<th>Thanatin</th>
<th>C-amidated thanatin</th>
<th>K18M</th>
<th>V16M</th>
<th>I14M</th>
<th>Y12M</th>
<th>G20R</th>
<th>G19Q</th>
<th>G18C</th>
<th>MSI-94</th>
<th>PGLa</th>
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<td>0.6-1.2</td>
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<td>1.2-2.5</td>
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<td>1-2</td>
<td>1.3-2.6</td>
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<table>
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<tr>
<th>Gram-negative bacteria</th>
<th>Thanatin</th>
<th>C-amidated thanatin</th>
<th>K18M</th>
<th>V16M</th>
<th>I14M</th>
<th>Y12M</th>
<th>G20R</th>
<th>G19Q</th>
<th>G18C</th>
<th>MSI-94</th>
<th>PGLa</th>
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<td>1.2-2.5</td>
<td>†</td>
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<tr>
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</table>

*MICs are expressed as the interval a–b, where a is the highest concentration tested at which bacteria are growing and b is the lowest concentration that causes 100% growth inhibition (15).

†The first and last residues refer to the N-terminal and C-terminal amino acids; numeral gives the number of amino acids.

No activity was detected at the highest concentration tested (40 μM), which corresponds to 10 times the endogenous concentration of thanatin in P. maculiventris.

The effect of thanatin on fungi was monitored by examining the development of spores. At high concentrations (10 μM and above), thanatin inhibited spore germination and no hyphae were observed for any of the strains tested. To get a further insight into the effect of thanatin on fungi, we incubated spores of the highly sensitive N. crassa in the presence of 0.6–1.2 μM thanatin. After 48 h, the medium containing the peptide was removed and replaced with fresh medium. Forty-eight hours later, the cultures were examined spectrometrically and microscopically. No growth recovery had occurred after replacement by fresh medium, indicating that thanatin is fungicidal. Similar results were obtained for all the species listed in Table 1.

Antimicrobial Activity of All-d-Thanatin. We have asked whether thanatin activity involves the stereospecific recognition of a chiral cellular target and we have synthesized all-d-thanatin. Two groups of results are apparent from Table 1: (i) all Gram-negative strains and three of the Gram-positive strains tested (B. subtilis, S. aureus, and P. acidolactici) are insensitive to all-d-thanatin or are sensitive at concentrations 1–2 orders of magnitude higher than those of native L-thanatin. (ii) Three strains of Gram-positive bacteria—A. viridans, M. luteus, and B. megaterium—are as sensitive to d-thanatin as to L-thanatin. All fungi are almost equally sensitive to the L- and d-enantiomers of thanatin.

Antimicrobial Activities of C-Amidated and Truncated Forms of Thanatin (Table 1). To analyze the possible contribution of the negative charge of the C terminus on the activity of thanatin, we synthesized the corresponding C-amidated peptide. C-amidated and native thanatin were active at similar concentrations on all bacterial and fungal strains tested, with the exception of P. aeruginosa, which was more sensitive (8-fold) to the C-amidated form.

To evaluate the structural features responsible for the antimicrobial activity of thanatin, we synthesized several truncated analogs. The results were as follows: (i) the absence of the three N-terminal Gly-Ser-Lys residues in molecule K18M does not noticeably affect the antibacterial activities, but it somewhat reduces antifungal activity. (ii) When the five most N-terminal residues are absent (V16M), the molecule retains good activity against several Gram-positive (A. viridans, M. luteus, B. megaterium) and Gram-negative (E. coli D22, E. coli D31, K. pneumoniae, and Enterobacter cloacae) bacteria but loses much of its efficiency against the others (B. subtilis and E. carotovora). Several Gram-negative bacteria (P. aeruginosa, S. typhimurium, and E. coli 1106) are insensitive at the concentrations tested (up to 40 μM). The activity also decreases against most of the fungal strains, with the exception of N. haematococca and A. brassicola. (iii) The absence of the seven N-terminal residues (I14M) severely reduces the activity against many of the bacteria and fungi. Exceptions are A. viridans and M. luteus, which are still very sensitive to this molecule. (iv) When the peptide is deprived of its nine N-terminal residues (Y12M), all activity is lost except against A. viridans, M. luteus, and B. megaterium, which are still affected by high concentrations of the truncated peptide. (v) Removal of the C-terminal residues (G20R, G19Q, G18C) strongly affects the activity of thanatin against Gram-negative bacteria. In fact, removal of the C-terminal methionine residue already has a marked effect. Gram-positive bacteria are sensitive to these C-terminally truncated isoforms, although at higher concentrations. This is also valid for fungi, which remain sensitive to reasonably high concentrations of these isoforms.

Finally, we have synthesized an isoform (I11C) consisting of the loop delineated by the disulfide bridge and extending N-terminally to the Ile-Ile-Tyr residue triplet. This isoform was tested against the most sensitive bacteria and fungi. A low but detectable activity (MIC 200–400 μM) was present against A. viridans and M. luteus (Table 2). However, the Gram-negative...
bacteria and the fungi were insensitive to this molecule at the highest concentration tested (400 μM).

**DISCUSSION**

We report the characterization of thanatin, a novel insect defense peptide with an exceptionally large activity spectrum. Thanatin has no sequence homology with other insect defense molecules but shows striking similarities with brevinins, a family of antimicrobial peptides isolated from frog skin secretions (4, 5, 8). The overall sequence homology between thanatin and brevinin-1 is close to 50% (Fig. 2) and both contain C-terminally a loop of 6 (thanatin) or 5 (brevinin) residues, delineated by an intramolecular disulfide bridge. This loop carries a strong positive charge in both molecules and contains a central threonine residue, which separates two subgroups of electropositivity (Arg-Arg or Lys-Lys vs. one Lys connected to either a Gly or an Ile residue). A similar motif—i.e., two C-terminally located cysteines flanking a group of charged residues separated by a Thr or a Ser residue—has been described, in addition to the brevinins, in other frog skin antimicrobial peptides (brevinins-2, esculetins, gaegurins, ranalexin) and has been dubbed the*_Rana*_ box (7). The insect box differs from the*_Rana*_ box by addition of the Asn residue within the loop. In both thanatin and brevinin-1, a cluster of six hydrophobic residues extends N-terminally up to a common Lys residue (positions 4 and 11). In all brevinins, the C-terminal residue corresponds to one of the two cysteines of the loop. In contrast, thanatin shows a C-terminal extension of three residues following this cysteine. As shown in our experiments with truncated isoforms, this triplet affects the activity of thanatin against certain microorganisms. Finally, in the frog peptides, the N-terminal domain connected to the C-terminal loop is systematically longer (ranging from 13 residues in ranalexin to 39 residues in some esculetins) than in thanatin (10 residues) and is believed to form an α-helix. The presence of two Pro residues in this domain of thanatin is not in favor of a similar structural organization.

Like brevinins and most of the related frog skin peptides, thanatin has a broad spectrum of activity against Gram-positive bacteria, Gram-negative bacteria, and fungi. This is in contrast to the hitherto characterized defense peptides of insects, none of which exhibits a similarly large activity spectrum at physiological concentrations (0.5–10 μM). Brevinins and related frog skin molecules are active against bacteria at concentrations similar to those of thanatin. In general, however, they appear less active against fungi than thanatin. Remarkably, though, some brevinins are active on yeast cells (8), in contrast to thanatin, which is inactive even at high concentrations. Thanatin is not hemolytic, which is also the case for brevinins (with the exception of brevinin-1E; see ref. 8).

The synthetic peptides and peptide fragments provide some insight into the structural requirements for the activity of thanatin. A minimum motif corresponding to the basic C-terminal loop linked to three residues on either the C-terminal (Y12M) or the N-terminal (I11C) side confers a low but detectable activity against the most sensitive Gram-positive strains (Tables 1 and 2). The concomitant presence of both the C-terminal and N-terminal three-residue extensions on the loop (I14M) confers a significant level of activity against several Gram-positive bacteria, Gram-negative bacteria, and fungi. This level of activity is increased when the N-terminal part is extended by the Val-Pro doublet (V16M). Finally, the two N-terminal residues Lys-Pro (K18M) increase the activity of the peptide against the less sensitive strains, and this isoform presents an activity spectrum identical to that of thanatin. In Table 2, we have compared the relative activities for the most sensitive strains of Gram-positive bacteria (*_A. viridans*_ and M. _luteus_), Gram-negative bacteria (*_E. coli*_ D31 and _E. coli*_ D22), and fungi (N. _crassa_). Only the complete sequence of thanatin confers full antifungal activity. The absence of the three (K18M) or five (V16M) N-terminal residues still allows for half to full activity against Gram-positive and Gram-negative bacteria. When the Val-Pro doublet on the N-terminal side is also absent, the level of activity drops dramatically against the Gram-negative cells (15%) and to a lesser extent against the Gram-positive strains (50%). The absence of the three C-terminal residues allows for some activity against Gram-positive bacteria (25%) and fungi (5%), but anti-Gram-negative activity is abolished (Table 2). These results point to four regions within thanatin that are essential for its full antimicrobial activity: (i) the C-terminal loop, (ii) the C-terminal three-residue extension, (iii) a stretch of seven N-terminal mostly hydrophobic residues, (iv) three additional N-terminal residues necessary for full antifungal activity but dispensable for antibacterial activity.

Thanatin is both bactericidal and fungicidal. Although the mode of action on bacteria is not yet understood, the results obtained suggest that the cytoplasmic membrane is not the target of the peptide and that thanatin, in contrast to insect defensins (16) and eceerpins (17), is not a pore-forming peptide. The rapid aggregation of thanatin-treated Gram-positive and Gram-negative cells is intriguing. Given the presence on thanatin of the 6-residue strongly basic loop, we propose that the peptide binds to the negatively charged surface of the bacteria (the lipopolysaccharide in the case of Gram-negative bacteria). Binding of the cationic peptide is expected to reduce the surface charge density of the lipopolysaccharide and thus to reduce the electrostatic repulsion between bacteria, allowing their aggregation.

The results obtained with all-D-thanatin strongly suggest that more than one mechanism of activity underlines killing of bacteria or fungi by thanatin. Indeed, several strains of Gram-positive bacteria and all fungal strains tested were equally sensitive to all-D-thanatin and to the all-L-enantiomer, which
argues against the existence of stereospecific recognition molecules on target cells. A similar situation is observed for such antimicrobial peptides as cecropins, magainins, and melitin where all-D molecules have the same activity as the natural all-L-enantiomers (18, 19). In marked contrast, however, all-D-thanatin is nearly inactive or poorly active against Gram-negative bacteria and several Gram-positive strains. These results, which support the idea that in these cells thanatin acts via a stereospecific receptor, are evocative of the data reported for the proline-rich 18-residue peptide apidaeacin from honeybee, the all-D-enantiomer of which is totally inactive against E. coli (20).

In conclusion, a septic injury induces in P. maculiventris synthesis of a 21-residue peptide, which at physiological concentrations exhibits the largest antimicrobial spectrum so far observed for any insect defense peptide. The structural data indicate that this molecule is homologous to various members of the brevinin family of frog skin antimicrobial peptides.

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