Isolation, Amino Acid Sequence, and Synthesis of Dermaseptin, a Novel Antimicrobial Peptide of Amphibian Skin†

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Received March 27, 1991; Revised Manuscript Received June 19, 1991

ABSTRACT: A 34-residue antimicrobial peptide named dermaseptin was purified to homogeneity from amphibian skin by a 3-step protocol involving molecular sieve filtration, ion-exchange chromatography, and reversed-phase high-performance liquid chromatography. The complete amino acid sequence of dermaseptin, ALWKTMLKKLGTMALHAGKAALGAAADTISQGTQ, was determined by automated Edman degradation of the peptide and of fragments generated by trypsin. Fast atom bombardment mass spectra of dermaseptin gave a protonated molecular ion m/z 3455.4 which matched the theoretical molecular weight predicted from the amino acid sequence. Dermaseptin was synthesized by the solid-phase method. The synthetic replicate was shown to be indistinguishable from natural dermaseptin with respect to chromatographic properties, amino acid sequence determination, and mass spectrometry analysis. Dermaseptin is a water-soluble, thermostable, and nonhemolytic peptide endowed with highly potent antimicrobial activity against pathogenic fungi at micromolar concentration. Circular dichroism spectra of dermaseptin in hydrophobic media indicated 80% α-helical conformation, and predictions of secondary structure suggested that dermaseptin can be configured as an amphiphatic α-helix spanning over residues 1–27, a structure that perturbs membrane functions regulating water flux.

The dermatous granular glands of amphibians synthesize and expel an extraordinarily rich variety of biologically active peptides (Erspamer, 1985; Erspamer & Melchiorri, 1980). This has allowed the characterization of more than 40 peptides, including thyrotropin-releasing hormone, angiotensins, and bombesin as well as the astonishing D-amino acid containing peptides dermorphin (Montecucchi et al., 1981), dermenkephalin[1] (Mor et al., 1989), and deltorphins I and II (Erspamer et al., 1989) that are provided with exceptional potency and selectivity toward the μ- or δ-opioid receptors (Erspamer et al., 1989; Kreil et al., 1989; Lazarus et al., 1989a,b; Sagan et al., 1989; Amiche et al., 1990). It is now well established that most of these amphibian peptides have counterparts, either identical or closely related peptides, in mammalian brain and the gastrointestinal tract (Erspamer, 1983; Spindel, 1986; Bevins & Zasloff, 1990). As a result of the repeated discovery of structural correspondence between amphibian skin peptides and mammalian neuropeptides or hormones, frog skin secretions are now considered as a model for the discovery of new mammalian peptides. Moreover, since the frog skin may contain up to 100 000 times larger amounts as compared with mammalian tissues, results obtained in the study of amphibian peptides are of interest in transcending comparative pharmacology and biochemistry as they contribute to the understanding of data assessed through investigations of mammalian counterparts.

It is commonly thought that the naked skin of frogs may take part in defensive secretions against predators and microbes. Early attempts to detect skin cytotropic substances have led to the discovery of the 24-residue hemolytic peptide bombinin from the skin secretions of *Bombina varietaga* (Csordas & Michl, 1970) (Table I). More recently, 2 highly homologous 23-residue antimicrobial peptides, named magainins, have been isolated from the skin of the African clawed frog *Xenopus laevis* (Zasloff, 1987) (Table I). These peptides share N-terminal sequence homology with bombinin and were shown to be active against microorganisms including Gram-positive and Gram-negative bacteria, yeast, and protozoa. The general design of bombinin is very similar to that of the magainins, revealing a high propensity to adopt α-helical conformation in hydrophobic media (Chen et al., 1988; Zasloff et al., 1988). The available data on the mode of action of the magainins suggest that they bind to the acidic components of

Table I: Hemolytic and Antimicrobial Peptides Isolated from the Frog Skin

Bombinin[a,b]	GIGA.L.SAKGALKGLAKGLAEHFAN-NH2
Magainin 1[b]	GIGGFLHSAKKFGKAFVGEIMKS
Magainin 2	GIGKFLHSAKKFGKAFVGEIMNS
XPF	GWASKIGQTLGKIAKVGLKELIQPK
PGLa	GMASKAGAIAGKIAKVALKAL-NH2
Dermaseptin	ALWKTMLKKLGTMALHAGKAALGAAADTISQGTQ

[a] Gaps (.) have been introduced to maximize homology with magainins. [b] Underlined residues represent identical amino acids between bombinin and magainins. The boxed regions represent identical amino acids among antimicrobial peptide subfamilies.[5]

† This work was supported in part by funds from the Centre National de la Recherche Scientifique, the Institut National de la Santé et de la Recherche Médicale (CRE 900206), and the Fondation Singer-Polignac.

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[1] Also referred to as deltorphin (Kreil et al., 1989) or dermorphin gene-associated peptide (Lazarus et al., 1989a).

0006-2960/91/0430-8824$02.50/0 © 1991 American Chemical Society

the bacterial membrane, inducing permeability changes (Matzusaki et al., 1989).

During purification of pro-dermorphin and related peptides from the skin extract of the South American arboreal frog *Phyllomedusa sauvagii* (Mor et al., 1991), we have detected an abundant, strongly basic peptide component. Inspection of the sequence of this 34-residue peptide suggested a high propensity to adopt an amphiphatic α-helix structure in hydrophobic media and resultant surface activity. In this paper, we describe the isolation, structural characterization, synthesis, and biological activity of this novel nonhemolytic antimicrobial peptide which demonstrated potent activity against pathogenic fungi. Because of its dermal origin and its antiseptic activity, this new antimicrobial peptide was named dermaseptin.

Materials and Methods

Purification of Dermaseptin from Frog Skin Extracts. Two *Phyllomedusa Sauvagii* dried skins (1 g per skin) were minced with scissors and extracted 3 times for 1 h each in 20 volumes (v/w) of 10% acetic acid at room temperature. Combined extracts were centrifuged for 30 min at 3000*g*, and the supernatant was lyophilized. The dried extract (0.2 g) was dissolved in 10% acetic acid and fractionated on a calibrated gel filtration column (Sephadex G-50 superfine, 1.5 × 100 cm) (Figure 1). Four-milliliter fractions were collected at a flow rate of 10 mL/h, from which aliquots were assayed by enzyme immunoassay (EIA)[2] as described by using antisera directed against the spacer peptide of pro-dermorphin, i.e., pro-dermorphin-[99–111] (Mor et al., 1991). Immunoreactive fractions that eluted in the mid-column (fractions 60–80) were pooled and evaporated under vacuum. This material was subjected to chromatography on a DEAE (Waters, Protein-Pak DEAE-5PW, 8 × 7.5 cm) column, equilibrated with 10 mM ammonium acetate, pH 6.8, and then eluted by a 10–400 mM ammonium acetate gradient. The strongly UV-absorbing material emerging before the start of the gradient was collected and lyophilized. An aliquot was dissolved in water containing 0.1% TFA and applied to a 5-μm C18 Licrosorb HPLC column (3.9 mm × 150 mm). Elution was achieved with an initial 5-min wash in 0.1% TFA/water and then with a 0–60% linear gradient of acetonitrile containing 0.07% TFA, at a flow rate of 1 mL/min. Absorbance was monitored at 280 nm. The major peak eluting at 53 min was further purified by reversed-phase HPLC developed with the same solvent system described above and with successively slower gradients of acetonitrile as described (Mor et al., 1989). The final purification step was performed on an analytical Delta-Pac (Waters, C18 5 μm, 150 mm × 2 mm) column. Elution was achieved with a linear gradient of 35–60% acetonitrile in 0.1% TFA/water for 25 min at a flow rate of 0.2 mL/min.

Trypsin Digestion. Sample was dissolved in 100 μL of water to which were added 300 μL of 50 mM Tris-HCl buffer, pH 8, containing 10 μg/mL trypsin (trypsin TPCK, Worthington) and 2 mM $CaCl_2$. The mixture was incubated overnight at 37 °C and then for 15 min at 100 °C. The resulting peptides were separated by reverse-phase HPLC on a C18 column (Waters, 5 μm, 150 mm × 2 mm) eluted with a 0–60% linear gradient of acetonitrile containing 0.07% TFA, at a flow rate of 0.2 mL/min.

Peptide Synthesis. Dermaseptin was prepared by stepwise solid-phase synthesis using fluoren-9-ylmethoxycarbonyl (Fmoc) polyamide active ester chemistry on a Milligen 9050 Pepsynthesizer. All Fmoc-amino acids were from Milligen. HMP-linked polyamide/Kieselguhr resin (Pepsin KA) and Fmoc-amino acid pentafluorophenyl (Pfp) and 3-hydroxy-2,3-dehydro-4-oxobenzotriazine (Dhbt) esters were from Milligen/Bioresearch. Side-chain protection groups used were *t*-Bu for Asp and Thr and *t*-Boc for Lys and His. Cleavage reaction of the peptide/resin was carried out at a concentration of 10 mg of peptide/resin in 1 mL of TFA/phenol (95/5% v/v) for 2 h at room temperature. After filtration to remove the resin and ether extraction, the crude peptide was purified by a combination of Sephadex gel filtration, ion-exchange chromatography, and preparative HPLC as described (Amiche et al., 1987). Homogeneity of the synthetic peptide was assessed by analytical HPLC, amino acid analysis, solid-phase sequence determination, and fast atom bombardment mass spectrometry (Nicolas et al., 1986).

Amino Acid Analysis. The amino acid composition was determined on a hydrolysate of the peptide (250 pmol) after phenylthiocarbamylation of the released residues by phenyl isothiocyanate followed by HPLC analysis as previously described (Nicolas et al., 1986).

Sequence Analysis. Sequence determination was carried out on an Applied Biosystem 470 gas-phase peptide sequenator. Phenylthiohydantoin amino acids were detected with an on-line Applied Biosystem 120A analyzer. Data collection and analysis were performed with an Applied Biosystems 900A module calibrated with 25 pmol of phenylthiohydantoin amino acid standards.

Alternatively, covalent sequence analysis was carried out on a Milligen 6600 solid-phase sequencer after covalent binding of the samples to Sequelon arylamine membranes. Phenylthiohydantoin amino acids were detected with an on-line HPLC column (Waters MS HPLC; SequaTag C-18 PTH analysis column, 350 × 3.9 mm) developed with ammonium acetate, pH 4.8, and acetonitrile and calibrated with 15 pmol of phenylthiohydantoin amino acid standards. Data collection and analysis were performed with a Maxima-PTH chromatography analysis software package (Dynamic Solution Corp., Division of Waters Chromatography, Milford, MA).

Fast Atom Bombardment Mass Spectrometry. Positive ion FAB spectra were recorded on a VG 70-250 instrument using a M-SCAN FAB gun operating at 8 kV and 1 mA as described (Nicolas et al., 1986). Xenon was used as the bombing gas. Peptide samples were dissolved in water, and 1–2 μL was loaded onto the probe tip previously coated with a glycerol/thioglycerol mixture. Spectra were recorded at 5-kV accelerating voltage, and ion mass assignment was achieved by peak matching to cesium iodide clusters.

Circular Dichroïsm Measurement. Peptide samples were dissolved in water (0.05–1 mg/mL) or in 20% trifluoroethanol/water (v/v). Spectra were obtained in a Jobin-Yvon Mark IV instrument linked to a Minc digital II miniprocessor, at room temperature using a quartz cuvette of 1- or 5-mm path length. CD data represent average values from five separate recordings. The contents of α-helix, β-sheet, and unordered structures were estimated as described (Yang et al., 1986).

Hemolysis and Coagulation Assays. Hemolytic activity was assayed with heparinated fresh rabbit blood rinsed 3 times in PBS (50 mM sodium phosphate/150 mM NaCl, pH 7) by centrifugation for 15 min at 20000 rpm. Red blood cells were then incubated at 37 °C in distilled water (positive control) or with various concentrations (0.8–1000 μg/mL) of synthetic dermaseptin in PBS. Release of hemoglobin was monitored as a function of time by measuring the optical density at 541

[2] Abbreviations: EIA, enzyme immunoassay; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid; FAB-MS, fast atom bombardment mass spectrometry; CD, circular dichroïsm; UV, ultraviolet; TFE, trifluoroethanol.

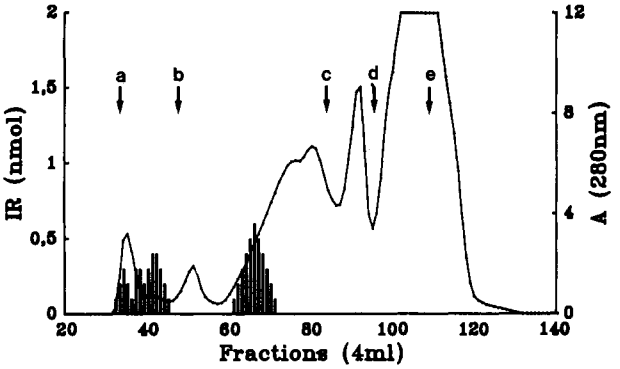

FIGURE 1: Size fractionation profile of the skin extract on a Sephadex G-50 (superfine) column (2.4 × 100 cm) using 10% acetic acid as eluant. Four-milliliter fractions were collected at a flow rate of 10 mL/h. The absorbance at 280 nm is represented as a straight line. Pro-dermorphin-[99–111] immunoreactivity (IR), represented in bars, was measured by an enzyme immunoassay (Mor et al., 1991) on aliquots of each fraction. Arrows point to the elution positions of some molecular weight markers: (a) bovine serum albumin; (b) trypsin inhibitor; (c) β-endorphin; (d) dermorphin; (e) potassium ferricyanide.

nm. For coagulation assays, fresh blood of guinea pig was collected in a citrated solution containing 4% tri-sodium citrate (10% v/v) and centrifuged for 5 min at 3000 rpm. The coagulation time of recalcified plasma (Howell's time) was determined at 37 °C after addition of 0.5 mL of plasma to 0.1 mg of dermaseptin in 0.5 mL of Tris containing 10 mM $CaCl_2$.

Assay of Antimicrobial Activity. Synthetic dermaseptin was tested for antimicrobial activity against one Gram-positive bacteria (*Bacillus subtilis*, IP 5262) and two pathogenic fungi (*Aspergillus fumigatus*, IP 864, and *Arthroderma simii*, IMI 101 693) by an inhibition zone assay on thin agar. A total of 1×10^6 bacteria or microconidia of the fungi was added to 10 mL of 0.5% agarose in Sabouraud glucose broth and poured over 90-mm petri dishes containing 20 mL of 2% agarose in Sabouraud broth. *Bacillus subtilis* cultures were incubated at 37 °C, and cultures of *Aspergillus fumigatus* and *Arthroderma simii* were incubated at 27 °C. Serial dilutions of dermaseptin were prepared in Sabouraud broth, and 10 μL was applied on 6-mm paper filters. Filters were then applied on the top surface of the solid agar, and inhibition zones around the filters were observed after overnight incubation at 37 or 27 °C, respectively. Morphological alterations were observed on *A. fumigatus* by using 48-h-old mycelium inoculated in 0.5 mL of Sabouraud glucose broth either in the absence or in the presence of 1–10–100 and 200 μg/mL synthetic dermaseptin. After 24-h incubation at 27 °C, the mycelium was examined on the light microscope.

Results

Purification of Dermaseptin. Dermaseptin was purified to homogeneity from *Phyllomedusa sauvagii* skin extracts by a three-step protocol including molecular sieve filtration, ion-exchange chromatography, and reverse-phase high-performance liquid chromatography. The elution profile on a calibrated Sephadex G-50 column of acidic extracts of *Phyllomedusa sauvagii* skin is shown in Figure 1. As reported previously (Mor et al., 1991), two immunoreactive materials recognized by antibodies directed against the spacer peptide of the dermorphin biosynthetic precursor were detected, one eluting near the void volume (fractions 30–50) and the other by the mid column (fractions 60–80). Authentic pro-dermorphin has been consequently purified to homogeneity from the first eluting peak by a combination of gel electrophoresis and reverse-phase HPLC (Mor et al., 1991). During the second step of purification of the late-eluting immunoreactive peak (fractions 60–80) achieved by anion-exchange chromatography (not shown), a strongly UV-absorbing non-immunoreactive peak accounting for 70% of the eluted material was found to emerge before the start of the gradient. Immunoreactivity corresponding to pro-dermorphin fragments eluted at an ammonium acetate concentration of about 0.3 M. Preliminary screening of the unretained nonimmunoreactive peak by determination of amino acid composition showed this basic peptide to be unrelated to pro-dermorphin.

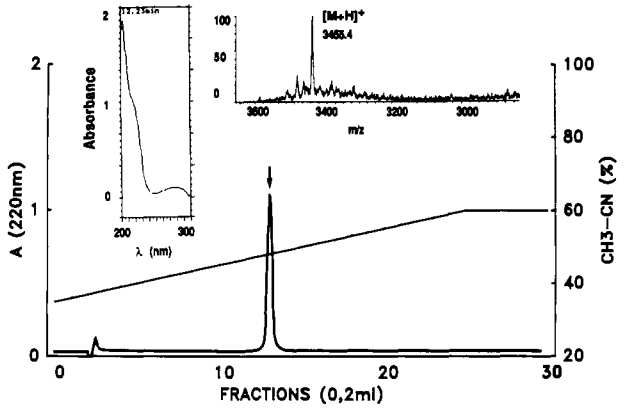

FIGURE 2: Final purification step of dermaseptin, performed on an analytical Delta-Pac (Waters, C18 5 μm, 150 mm × 2 mm) column. Elution was achieved with a linear gradient of 35–60% acetonitrile in 0.1% TFA/water for 25 min at a flow rate of 0.2 mL/min. The arrow points to the elution position of synthetic dermaseptin under the same conditions. The left-hand inset represents the UV absorbance spectrum of the eluting peak at 12.23 min. The right-hand inset represents the quasi-molecular ion region of the positive FAB mass spectrum of purified dermaseptin.

Table II: Amino Acid Composition of Natural Dermaseptin

amino acid	residues/mol: from acid hydrolysate[a]	residues/mol: from amino acid sequences
Asx	1.2 (1)	1
Glx	2.1 (2)	2
Ser	1.1 (1)	1
Thr	3.5 (3–4)	4
Gly	4.4 (4)	4
His	0.9 (1)	1
Ala	7.6 (8)	8
Met	1.9 (2)	2
Leu	4.8 (5)	5
Ile	0.8 (1)	1
Trp	ND[b]	1
Lys	3.9 (4)	4
	total 32–33	total 34

[a] Calculated by assuming a molecular weight of 3500. Integral values are given in parentheses. [b] ND, not determined.

The basic peptide was subsequently purified by using a series of HPLC runs operating with successively slower gradients of acetonitrile in 0.1% TFA/water. During the final step of purification, the peptide emerged as a symmetrical sharp peak (Figure 2) accounting for 96% of the eluted material. Typically, the amount of purified dermaseptin recovered was 40 μg starting from 1 g of dried skin. This purified material was used for further chemical and biological analysis.

Primary Structure of Dermaseptin. The amino acid composition of natural dermaseptin determined after acid hydrolysis is reported in Table II. The peptide was calculated to contain about 32 amino acid residues with no estimation of tryptophan. As expected from its behavior during ion-exchange chromatography and reverse-phase HPLC, dermaseptin is rich in basic and hydrophobic residues. Moreover, examination of the near-UV spectra of natural dermaseptin

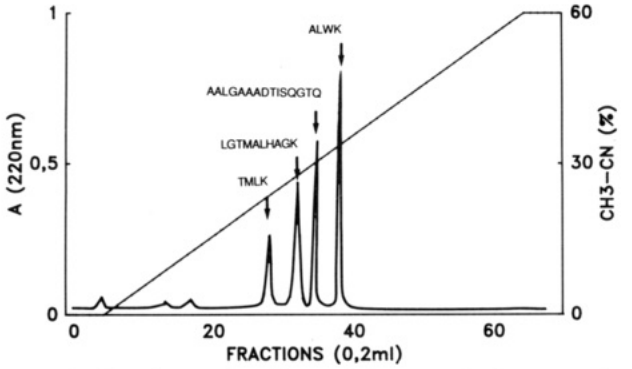

FIGURE 3: Tryptic mapping of natural dermaseptin after separation by reverse-phase HPLC on a C18 column (Waters, 5 μm, 150 mm × 2 mm). Elution was with a 0–60% linear gradient of acetonitrile containing 0.07% TFA at a flow rate of 0.2 mL/min. The amino acid sequences of the recovered tryptic peptides are shown in single-letter code. Arrows point to the elution positions of the corresponding fragments obtained after trypsin digestion of synthetic dermaseptin.

(inset of Figure 2) indicated the presence of, at least, one residue of tryptophan per mole which has been destroyed during acid hydrolysis.

The sequence of dermaseptin was determined up to the 34th residue as ALWKTMLKKLGTMALHAGKAALGAA-ADTISQGTQ by automated Edman degradation with a gas-phase sequencer. The amino acid composition predicted by sequence analysis was in excellent agreement with the amino acid analysis and spectral properties of natural dermaseptin. A thorough computer search[3] comparing the dermaseptin sequence to all published protein sequences revealed no significant homology to any prokaryotic or eukaryotic protein.

Since the sequence analysis does not yield information on the presence of a C-terminal amide group or other post-translational modifications of amino acid side chains, natural dermaseptin was further subjected to fast atom bombardment mass spectrometry. As shown in Figure 2, a strong unequivocal pseudomolecular ion $[M + H]^+$ was observed at m/z 3455.4 in the positive ion mode. This value corresponded precisely to that expected theoretically for the proposed amino acid sequence of the peptide.

Further support for the proposed structure was obtained through trypsin digestion of the purified peptide followed by amino acid sequence identification of the released fragments after HPLC separation. As expected from inspection of the proposed sequence, tryptic digestion of dermaseptin gave rise to four major cleavage products (Figure 3). The primary structure of each fragment was determined by automated Edman degradation and shown to correspond precisely to that expected from the proposed structure of the whole peptide.

Solid-Phase Synthesis of Dermaseptin. A definitive confirmation of the structure proposed for dermaseptin was obtained through solid-phase synthesis of the peptide using Fmoc methodology. Purification of the synthetic peptide was by gel filtration, ion-exchange chromatography, and reverse-phase HPLC. After purification, synthetic dermaseptin was shown to be indistinguishable from natural dermaseptin by the following chemical and physical criteria. The purified synthetic peptide showed by HPLC a unique sharp peak eluting at the position of the corresponding natural product (Figure 2) both under gradient or under isocratic conditions. The sequence

[3] Protein Sequence Query Program of the Protein Identification Resource (PIR), registered mark of the National Biomedical Research Foundation (NBRF), Georgetown University Medical Center.

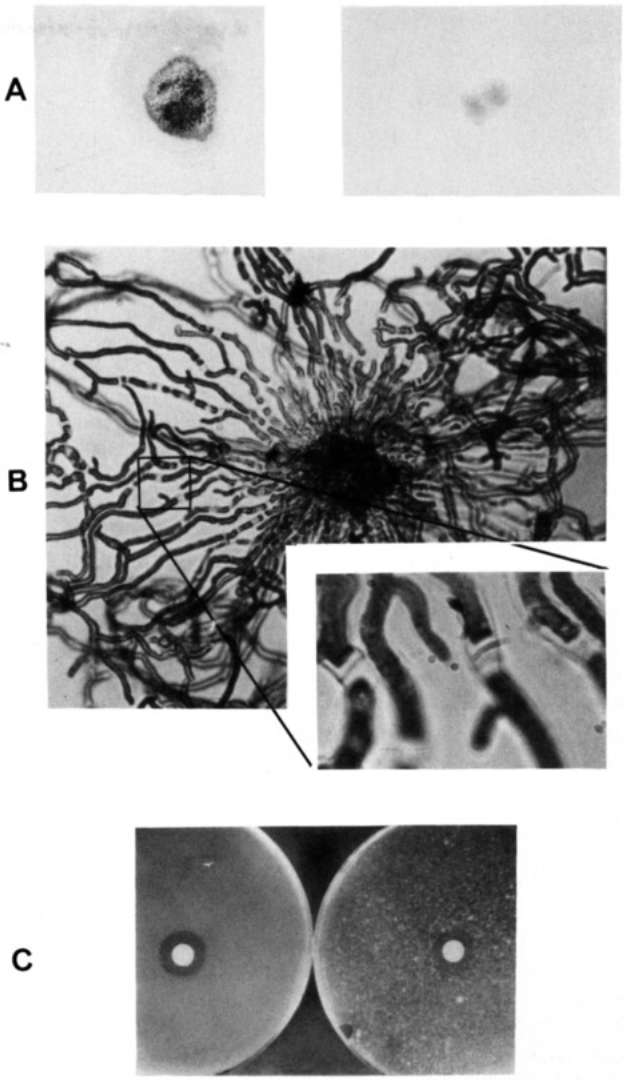

FIGURE 4: Antimicrobial activity of dermaseptin. (A) Inhibition of growth obtained in a 48-h-old *Aspergillus fumigatus* culture exposed for 24 h to dermaseptin in Sabouraud glucose broth. Cultures were incubated at 27 °C in the absence (left) or in the presence (right) of 10 μg/mL synthetic dermaseptin. (B) Morphological alterations observed from (A) in the light microscope (70×) indicating that hyphae of *A. fumigatus* are altered after exposure to dermaseptin. The cytoplasm is contracted, empty spaces appear in the fungal cell, and no conidium is seen. (C) Classical inhibition zone assay (10 μg/mL synthetic dermaseptin) on thin agar against *Arthroderma simii* (left) and *Aspergillus fumigatus* (right). Cultures were incubated at 27 °C.

of synthetic dermaseptin was determined up to the last residue by automated Edman degradation with a solid-phase sequencer. It was found to be identical with that obtained from the natural product. Mass spectrometry of a sample of synthetic dermaseptin gave a strong quasi-molecular ion $[M + H]^+$ appearing in the positive ion mode at m/z 3455.4; this value corresponded exactly to that obtained from natural dermaseptin. Final confirmation of the structural identity between natural and synthetic dermaseptin was achieved by submitting the synthetic peptide to digestion with trypsin. The digest was analyzed by HPLC and proved to be identical with that generated from natural dermaseptin (Figure 3).

Since synthetic dermaseptin was found to be indistinguishable from its natural counterpart, it was used in the following to study the mode of action and the conformation of dermaseptin.

Antimicrobial Activity of Dermaseptin. Synthetic dermaseptin was tested for antimicrobial activity against two pa-

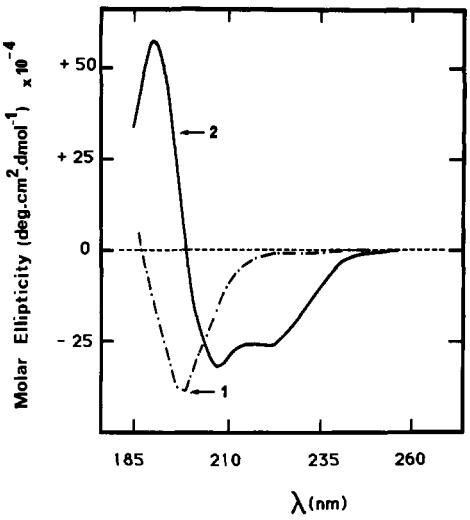

FIGURE 5: Circular dichroïsm spectra of dermaseptin (0.5 mg/mL) in water in the absence or the presence of trifluoroethanol. Observed spectra: curve 1, in water, is characteristic of an unordered structure; curve 2, in the presence of 20% TFE, the peptide becomes structured, exhibiting a high level of helical folding.

thogenic fungal species (*Aspergillus fumigatus* and *Arthroderma simii*) and one Gram-positive bacteria (*Bacillus subtilis*). A classical inhibition zone assay on thin agar was used. As seen in Figure 4C, 10 μg/mL dermaseptin suppressed fungal but not bacterial growth on solid medium. When 48-h-old *A. fumigatus* or *A. simii* were exposed to dermaseptin at 10 μg/mL in Sabouraud glucose broth, a similar inhibition of growth was noted (Figure 4A) after 24-h incubation at 27 °C. Observation of the mycelium under the light microscope indicated clearly that the hyphae were altered (Figure 4B). The cytoplasm was contracted, and empty spaces appeared in the fungal cells. In addition, whereas a 3-day-old untreated culture of *Aspergillus fumigatus* developed a typical green color due to the presence of conidia, no conidium was seen in the white dermaseptin-treated cultures.

Secondary Structure of Dermaseptin. Prediction of the secondary structure of dermaseptin according to the method of Chou and Fasman (1978) identified no less than 79% helical zone and 21% unordered structure. Tentative localization of the helix indicated a domain spanning residues 1–27 ($\langle P_\alpha \rangle$ = 1.13). Information theory (Garnier et al., 1978) predicted 79% helix (residues 1–27), 6% β-sheet (residues 28–29), and 15% coil while the neural networks method (Qian & Sejnowski, 1988) indicated 72% helix (residues 2–26) and 28% unordered structure. Figure 5 illustrates the far-UV circular dichroïsm spectra of synthetic dermaseptin in the absence and presence of trifluoroethanol. The content of α-helix, β-sheet, and unordered structure was calculated according to Yang et al. (1986). The CD spectra of dermaseptin in water were characteristic of an unordered structure with 0% helical content. In the presence of 20% trifluoroethanol, the peptide became structured, exhibiting a high level, i.e., 77%, of helical folding (θ_{222} = 26.7 × 10^4 deg cm^2 dmol^{-1}) and 23% unordered structure. No concentration dependency was observed in the range 10–200 μM.

Discussion

The present study described the isolation, characterization, and synthesis of dermaseptin, a novel antimicrobial peptide present in the frog skin. Dermaseptin was purified to homogeneity by a three-step protocol involving molecular sieve filtration, ion-exchange chromatography, and reverse-phase HPLC. Homogeneity of the final preparation was assessed by amino acid analysis, automated amino acid sequence determination, tryptic mapping, mass spectrometry analysis, and solid-phase synthesis. Dermaseptin was established as a 34-residue peptide (Table I) by automated Edman degradation of the whole purified peptide. The agreement between the amino acid composition and the amino acid sequence indicated that the full sequence had been obtained. Positive ion fast atom bombardment mass spectrometry identified a molecular ion with average mass of 3455. This value corresponded to the theoretical mass based upon the proposed amino acid sequence and indicated the absence of any additional post-translational modifications (carboxamidation, O- and/or N-glycosylation, phosphorylation) of the constitutive amino acid side chains. Additional support of the proposed structure was obtained through trypsin digestion of purified dermaseptin followed by amino acid sequence determination of the four released fragments after HPLC separation. Definitive confirmation of the proposed structure was achieved through solid-phase synthesis of dermaseptin. Synthetic dermaseptin showed chemical and physical properties that were indistinguishable from those of the natural compound. The identities of the natural and synthetic products confirm that the active natural peptide does not contain additional side-chain modification and that antimicrobial activity exhibited by dermaseptin reflects intrinsic properties.

Inhibition zone assays have shown[4] that dermaseptin presents potent activity against pathogenic fungi (*Aspergillus fumigatus* and *Arthroderma simii*) at micromolar concentrations. Whereas magainins also showed potent antimicrobial activity against bacteria and yeast, dermaseptin is the first peptide isolated from frog skin which developed inhibition of growth of a pathogenic mold and morphological alteration of its hyphae. Experiments are underway to determine the precise spectrum of antibiotic activity of dermaseptin, especially against pathogenic fungi since, unlike antibacterial products, there are only a few agents developed in the therapy of fungal infections.

An examination of the sequence of dermaseptin shows the amino-terminal half of the molecule is strongly basic and contains long alternating hydrophobic stretches. Only the C-terminal region, residues 27–34, can be regarded as hydrophilic and negatively charged. Prediction of secondary structures according to Chou and Fasman (1978) showed 20% coil and (P_α) values = 1.13 for a large domain spanning residues 1–27, i.e., 80% of the total residues. Similar results were obtained by using information theory (79% α-helix, 6% β-sheet, 15% coil) (Garnier et al., 1978) or the neural networks method (72% helix, 28% coil) (Qian & Sejnowski, 1988). When plotted as an α-helical wheel (Schiffer & Edmunson, 1967), hydrophobic and hydrophilic domains are clearly distinguishable (Figure 6). In this conformation, there are 10 hydrophilic or charged residues on 1 side of the cylindrical surface and 12 hydrophobic residues with no charged residue on the opposite side. The polar face of dermaseptin subtends an average angle of 160° perpendicular to the long axis of the helix. Interestingly, the lysyl residues are not clustered but distributed over the surface of the polar side. The value of the mean angle subtended by positively charged residues is 150°. The only residues that appear not to fit the regular pattern of amphiphilicity are those of the C-terminal end, i.e., residues 28–34. This suggests that dermaseptin can be configured as an amphiphatic α-helix spanning over residues 1–27.

[4] A detailed account of the antimicrobial spectrum of activity of dermaseptin will be published elsewhere.

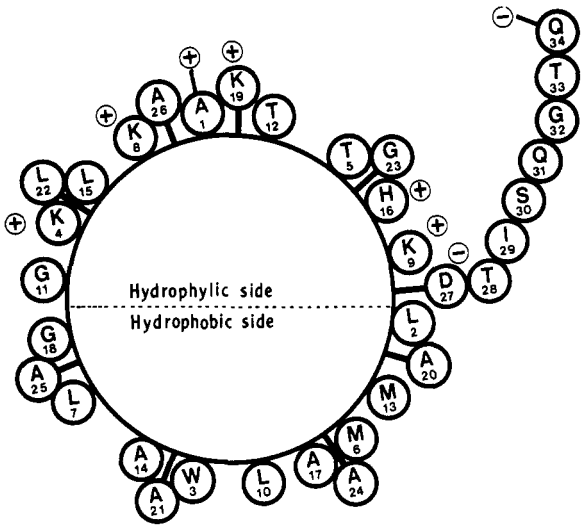

FIGURE 6: α-Helical wheel plot of dermaseptin. Helical wheel projection for dermaseptin residues 1–27 shows its amphiphatic structure. In this conformation, periodic variation in the hydrophobicity value of the residues along the peptide backbone with a 3.6 residues per cycle period characterizes an α helix. A hydrophobic domain and a charged hydrophilic domain are clearly distinguishable on each side of the cylindrical surface. The only residues that do not fit these domains are those of the C-terminal end, residues 28–34.

Support of this proposal was obtained through CD measurements of dermaseptin either in water or in hydrophobic media. The CD spectra of dermaseptin in water were characteristic of a nonstructured conformation. In the presence of a low concentration of a helix-promoting solvent, however, dermaseptin became structured with a rather high level, i.e., 77%, of α-helix conformation. Hence, both theoretical predictions and CD measurements suggest that dermaseptin can form a nearly perfect amphiphatic α-helix in hydrophobic media.

Since α-helical amphiphatic peptides may be membrane-disruptive, dermaseptin was assayed for hemolytic activity against rabbit erythrocytes. Whereas 100% hemolysis was observed after 20-min incubation with 1000 μg/mL dermaseptin, no hemolysis could be detected up to 200 μg/mL after 2-h incubation, nor did dermaseptin present any detectable effect on the normal coagulation time (120 s) at concentrations ranging from 0.8 to 1000 μg/mL (not shown).

Although the mechanism of action of dermaseptin remains to be shown, it is probable that the antimicrobial, nonhemolytic activity of this peptide is due in part to surface-active properties and disturbance of membrane functions. Since dermaseptin is a cationic peptide that can form a long amphiphatic helix in hydrophobic media, it is tempting to suggest that it could interact with phospholipid bilayers, thus interfering with functions governing water flux. Since 20 residues in a α-helical structure are the minimum length required to span a lipid bilayer, this could suggest that dermaseptin perturbs water flow by forming transmembrane channels. Synthetic peptides truncated from the amino-terminal or the C-terminal ends are needed to further explore this hypothesis.

Dermaseptin represents a new member of a growing family of vertebrate nonhemolytic antimicrobial peptides including the magainins, the 21-residue peptide PGL_a, and a closely related 25-residue peptide, XPF, derived from processing of the xenopsin biosynthetic precursor (Hoffman et al., 1983; Sures & Crippa, 1984; Richter et al., 1985; Gibson et al., 1986; Soravia et al., 1988) (Table I). These peptides are small-sized (21–34 residues), strongly basic, and potentially amphiphatic and exhibit a distinct spectrum of antibiotic activity. These features characterize all the hitherto known vertebrate antimicrobial peptides synthesized by the skin and suggest that they act via similar mechanisms to permeabilize microbial membranes despite striking differences in their primary structure.[5] These differences may contribute to the variability in antimicrobial activity, particularly with respect to differences in the spectrum of antibiotic action. Since dermaseptin has no sequence identity with the known amphibian peptide antibiotics—PGL_a/XPF and magainin 1/magainin 2—it should be a useful tool for identifying key features and conformational determinants responsible for membrane permeabilization.

The relatively recent detection of antibiotic peptides in vertebrates, despite their abundance, underlines the limited extent to which the various mechanisms of innate resistance to infections have been explored. Moreover, since frog skin peptides have structural correspondents in mammals, a careful search might uncover additional endogenous antibiotic molecules and contribute to further illuminate our understanding of the molecular mechanisms of antimicrobial warefare.

ACKNOWLEDGMENTS

We thank P. Y. Haumont (Milligene, Millipore S.A., France) for synthesis of a sample of dermaseptin, Dr. G. Ricart (Lille) for mass spectra, and P. Paroutau (Applied Biosystems, France) for gas-phase sequence analysis. The expert assistance of Drs. J. Maillard, P. Laugâa, M. Naïm, and J. J. Montagne is acknowledged.

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[5] Since magainin 2 differs from magainin 1 by only two substitutions and since PGL_a and XPF show extensive sequence homology, magainins 1 and 2 and PGL_a and XPF should be considered respectively as closely related members of two distinct subfamilies of antimicrobial peptides.

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Amino Acid Sequence Modulation of Gramicidin Channel Function: Effects of Tryptophan-to-Phenylalanine Substitutions on the Single-Channel Conductance and Duration†

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Received February 21, 1991; Revised Manuscript Received June 3, 1991

ABSTRACT: Linear gramicidins with one, two, or three Trp → Phe substitutions in the gramicidin A sequence form $\beta^{6.3}$-helical channels that have widely varying conductances and average durations. The variations in single-channel conductance and average duration are uncoupled. The single-channel conductance decreases as a monotonic function of the number of Trp → Phe substitutions, and the relative conductance decrease induced by a given Trp → Phe substitution is only weakly affected by substitutions at other positions. These results suggest that each Trp influences the conductance independently, most likely through electrostatic interactions between the Trp dipole(s) and the permeant ion (as was deduced previously for aromatic side-chain substitutions at position one [Koeppe, R. E., Mazet, J.-L., & Andersen, O. S. (1990) *Biochemistry 29* (2), 512–520]). Trp → Phe substitutions exert a complex, nonadditive influence on average duration as well as the energetics of heterodimer formation. These changes are presumably due to sequence-specific differences in the channel's surface chemistry—which may be related to ability of the Trp indole NH moieties to form hydrogen bonds with the lipid backbone oxygens and/or interfacial H_2O.

Two central questions in molecular biophysics are how the function of a membrane-spanning ion channel is determined by the channel's conformation and how both conformation and function are governed by the primary sequence. In order to address these questions, it is necessary not only to know a channel's conformation but also to understand the relationship between the physicochemical characteristics of individual amino acid side chains and the energetics of channel formation and ion permeation. We will in this article examine these general questions using the linear gramicidins as a family of prototypic channel formers.

The linear gramicidins are well suited for elucidating the relations that exist between structure and function because the general channel structure is known (see below); the channels are selective for monovalent cations, single-channel experiments thus allow for precise measurements of channel function; individual side chains can be altered readily by using peptide chemical methods (Mazet et al. 1984); and the structural and functional consequences of a sequence substitution can be evaluated (Russell et al., 1986; Durkin et al., 1990; Koeppe et al., 1990).

The amino acid sequence for [Val^1]gramicidin A (gA) is (Sarges & Witkop 1965)

† This work was supported by the United States Public Health Service through NIH Grants GM 21342, GM 34968, and RR 07101. M.D.B. was supported by a Cornell University Medical College M.D./Ph.D. fellowship.

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0006-2960/91/0430-8830$02.50/0 © 1991 American Chemical Society