Hayes, F. N., & Mitchell, V. E. (1969) *J. Chromatogr. 39*, 139–146.
Hoard, D. E., Ratliff, R. L., Williams, D. L., & Hayes, F. N. (1969) *J. Biol. Chem. 244*, 5368–5373.
Hoopes, B. C., & McClure, W. R. (1981) *Nucleic Acids Res. 9*, 5493–5604.
Horrocks, D. L. (1977) *Beckman Technical Report 1095-NUC-77-IT*, Beckman Instrument Co., Palo Alto, CA.
Inman, R. B. (1964) *J. Mol. Biol. 9*, 624–637.
Langridge, R., & Rich, A. (1963) *Nature (London) 198*, 725–728.
Lee, J. S., Johnson, D. A., & Morgan, A. R. (1979) *Nucleic Acids Res. 6*, 3073–3091.
Lomant, A. J., & Fresco, J. R. (1963) *Prog. Nucleic Acid Res. Mol. Biol. 15*, 185–218.
Marck, C., Thiele, D., Schneider, C., & Guschlbauer, W. (1978) *Nucleic Acids Res. 5*, 1979–1996.
Marsh, R. E., Bierstedt, R., & Eichhorn, E. L. (1962) *Acta Crystallogr. 15*, 310–316.
Morden, K. M., Chu, Y. G., Martin, F. H., & Tinoco, I., Jr. (1983) *Biochemistry 22*, 5557–5563.
Patrick, M. H., & Gray, D. M. (1976) *Photochem. Photobiol. 24*, 507–513.
Setlow, R. B., Carrier, W. L., & Bollum, F. J. (1965) *Proc. Natl. Acad. Sci. U.S.A. 53*, 1111–1118.
Tautz, D., & Renz, M. (1984) *J. Mol. Biol. 172*, 229–235.
Thiele, D., Marck, C., Schneider, C., & Guschlbauer, W. (1978a) *Nucleic Acids Res. 5*, 1997–2012.
Thiele, D., Sarocchi, M.-T., Guschlbauer, W., Lezius, A., & Marck, C. (1978b) *Mol. Biol. Rep. 1*, 155–160.
Varghese, A. J. (1971) *Biochemistry 10*, 2194–2199.
Westhof, E., & Sundaralingham, M. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 1852–1856.

N-Terminal Analogues of Cecropin A: Synthesis, Antibacterial Activity, and Conformational Properties†

David Andreu* and R. B. Merrifield
Rockefeller University, New York, New York 10021
Håkan Steiner and Hans G. Boman
Department of Microbiology, University of Stockholm, S-106 91 Sweden
Received August 15, 1984

ABSTRACT: Six analogues of the 37-residue antibacterial peptide cecropin A were synthesized by the solid-phase method: cecropin A-(2–37), [Glu^2]cecropin A, [Pro^4]cecropin A, [Glu^6]cecropin A, [Leu^6]cecropin A, and [Pro^8]cecropin A. Their antibacterial activities against four test organisms were determined and related to conformational changes observed in their CD spectra and were discussed on the basis of a previously proposed amphipathic α-helix model. An aromatic residue in position 2 was shown to be important for activity against all tested bacteria. The highly α-helical 1–11 region of cecropin A did not appear to play a significant role in its activity against *Escherichia coli* but was clearly involved in its interaction against *Pseudomonas aeruginosa*, *Bacillus megaterium*, and *Micrococcus luteus*.

The induction of immunity in the pupae of the North American silk moth *Hyalophora cecropia* elicits a powerful antibacterial response characterized by the appearance of the cecropins, and of several other immune proteins in the insect hemolymph (Boman & Steiner, 1981). Cecropin A, a major contributor to this induced immunity, was recently synthesized by us (Andreu et al., 1983). Its antibacterial activity had been previously discussed in terms of a model consisting of two α-helical segments extending from the N-terminus to residue Ala^{22} and from residue Ala^{25} to the C-terminus with a major disruption of the helix caused by residues Gly^{23} and Pro^{24} (Merrifield et al., 1982). This model could be adjusted to offer a distribution of charged and hydrophobic residues that closely conformed to an ideal amphipathic helix (Segrest et al., 1974; Assman & Brewer, 1974; Kaiser & Kezdy, 1984) (Figure 1). We had also previously shown that both N- and C-terminal regions are needed to confer specificity of action to cecropin A. In particular, a considerable loss of activity against most of the selected test organisms was detected when the two N-terminal residues of cecropin A (Lys^1, Trp^2) were removed (Andreu et al., 1983).

In order to test the proposed model and thus achieve a better understanding of the mechanism of action of cecropin A, we have prepared six analogues having single-residue modifications: cecropin A-(2–37), [Glu^2]cecropin A, [Pro^4]cecropin A, [Glu^6]cecropin A, [Leu^6]cecropin A, and [Pro^8]cecropin A. The N-terminal region has been selected both for its synthetic convenience and for its already established role in the specificity of the molecule. The six synthetic analogues have been tested against two Gram-negative and two Gram-positive organisms, and their antibacterial activity is discussed and related to the conformational changes introduced in the molecules.

Experimental Procedures

Materials. Benzhydrylamine hydrochloride resin (0.56 mmol of N/g) was purchased from Beckman, Palo Alto, CA.

† This research was supported by Grant AM 01260 from the U.S. Public Health Service (to R.B.M.) and by Grant B2453 from the Swedish Natural Science Research Council (to H.G.B.). D.A. was supported by a postdoctoral fellowship from the Fundación Juan March, Spain, and by a grant from KabiGen AB, Sweden.

0006-2960/85/0424-1683$01.50/0

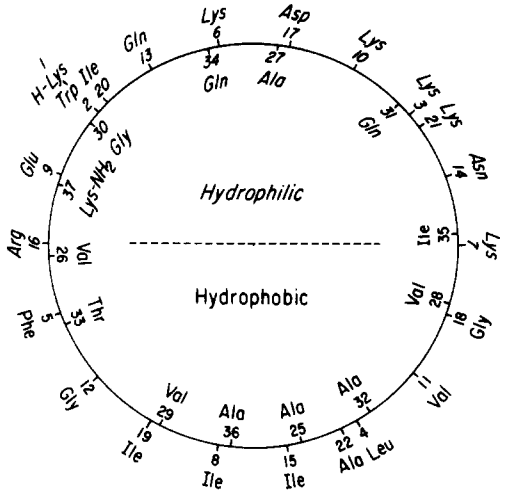

FIGURE 1: Amphipathic α-helix model proposed for cecropin A [from Merrifield et al. (1982)].

tert-Butyloxycarbonyl (Boc) amino acids were from Peninsula Laboratories. The main solvents were dichloromethane (Fisher), distilled over anhydrous Na_2CO_3, *N*,*N*-dimethylformamide (DMF) (MCB Chemical), stored over 4-Å molecular sieves, and HPLC-grade acetonitrile (Fisher). Other reagents were trifluoroacetic acid (TFA) (Halocarbon), *N*,*N*-diisopropylethylamine (DIEA) (Aldrich), distilled from CaH_2 before use, *N*,*N*′-dicyclohexylcarbodiimide (DCC) (Fluka), 1-hydroxybenzotriazole (HOBt) (Aldrich), recrystallized from 80% ethanol, *p*-cresol (Aldrich), *p*-thiocresol (Fluka), dimethyl sulfide (Fluka), and anhydrous HF (Matheson).

General Methods. Hydrolyses of free peptides were done with 6 N HCl in evacuated, sealed tubes at 110 °C, 24 h (Crestfield et al., 1963). Peptide–resins were hydrolyzed in 12 N HCl/propionic acid (1:1) at 130 °C, 6 h (Westall et al., 1970). After filtration, hydrolysates were analyzed on a Waters high-pressure liquid chromatographic (HPLC) instrument equipped with a fluorescence detector Model 420 AC and a Wisp 710 B automatic sample injector, using an Altex Ultrasphere ODS (4.6 × 250 mm) column. HF reactions were carried out in a Diaflon HF apparatus (Toho Kasei, Osaka, Japan). Gel filtration was done on a 2 × 100 cm Sephadex G-25 column, eluted with 1 M HOAc at 18 mL/h. Carboxymethylcellulose (CM-52, Whatman) chromatography was done on a 1 × 20 cm Altex column, equilibrated with 0.1 M ammonium formate, pH 6.6 (ca. 10 mS). Elution was with a linear ammonium formate gradient, at pH 6.6, from 0.1 to 0.8 M (ca. 65 mS) for peptides with 7+ net charge and from 0.1 to 0.6 M (ca. 50 mS) for peptides with 6+ net charge (250 mL of each solution in both cases), at 18 mL/h. Preparative reverse-phase liquid chromatography was done on C_{18} silica (10 μm, Waters Associates) packed in a 2.2 × 13 cm Michel-Miller column (Ace Glass), equilibrated with 5% acetonitrile in 0.05% TFA in water. The samples were loaded, and the column was washed for 60 min. Elution was then performed with a linear 20–40% gradient of acetonitrile in 0.05% TFA in water over a period of 14 h, at 1 mL/min. Analytical HPLC was performed at room temperature on a μBondapak C_{18} reverse-phase column (4 × 300 mm, Waters Associates) in a Waters instrument fitted with a Schoeffel variable-wavelength UV detector. Solution A contained 950 mL of water, 50 mL of acetonitrile, and 0.445 mL of TFA; solution B contained 350 mL of water, 650 mL of acetonitrile, and 0.385 mL of TFA. Linear gradients in the 30–70% range for 30 min, at 2 mL/min flow rate, were used. Detection was at

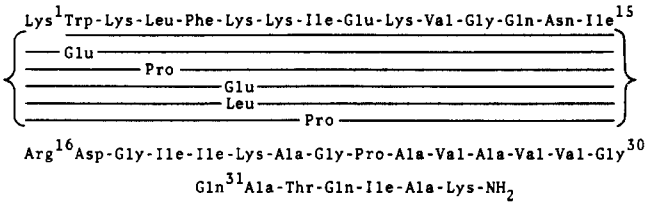

FIGURE 2: Amino acid sequences of the six synthetic cecropin A analogues.

210 nm. Polyacrylamide gel electrophoresis was performed in 15% polyacrylamide gels, in sodium acetate buffer at pH 4.0. Gels were stained for protein with Coomassie brilliant blue.

Synthetic Protocols. Synthesis was performed manually in a reaction vessel that has been previously described (Merrifield et al., 1982). The following standard double-coupling protocol was used on 2.5 g of starting resin (0.50 mmol/g) for each synthetic cycle, with the exceptions described under Results: (1) 50% TFA–CH_2Cl_2 (50 mL, 2 times 1 min); (2) 50% TFA–CH_2Cl_2 (50 mL, 1 times 20 min); (3) CH_2Cl_2 (50 mL, 6 times 1 min); (4) 5% DIEA–CH_2Cl_2 (50 mL, 2 times 2 min) (for C-terminal Lys residue, steps 1 and 2 were omitted and step 4 was 2 times 10 min); (5) CH_2Cl_2 (50 mL, 6 times 1 min); (6) Boc amino acid (3 equiv) in CH_2Cl_2 (15 mL) added to reaction vessel, rinsed with CH_2Cl_2 (5 mL), and shaken at room temperature for 5 min; (7) DCC (3 equiv) in CH_2Cl_2 (3 mL) added to reaction vessel, rinsed with CH_2Cl_2 (2 mL), and shaken for 60 min; (8) CH_2Cl_2 (50 mL, 4 times 1 min); (9) 5% DIEA–CH_2Cl_2 (50 mL, 2 min); (10) CH_2Cl_2 (50 mL, 4 times 1 min); (11) DMF (50 mL, 2 times 2 min), (12) symmetric anhydride (0.5 equiv) in CH_2Cl_2–DMF (1:1 v/v) (20 mL) added to reaction vessel at 0 °C, rinsed with CH_2Cl_2–DMF (1:1) (5 mL), and shaken for 60 min at room temperature; (13) DMF (50 mL, 2 times 2 min); (14) CH_2Cl_2 (50 mL, 4 times 1 min); (15) 5% DIEA–CH_2Cl_2 (50 mL, 1 times 2 min); (16) CH_2Cl_2 (50 mL, 4 times 1 min); (17) ninhydrin test. After 20 cycles, and due to the great swelling of the deprotected polymer after step 3, step 4 was modified to be 10% DIEA–CH_2Cl_2 (50 mL, 3 times 5 min), in order to ensure a complete neutralization.

Circular Dichroism Measurements. Spectra were obtained in a Jasco instrument, at room temperature. Peptide samples were dissolved in 2.5 mM phosphate buffer at pH 7.4 (0.05–0.1 mg/mL) and examined. Increasing amounts of hexafluoro-2-propanol were then added to each sample to make up a total 20% organic solvent concentration.

Antibacterial Activity Assays. Thin agar plates containing rich medium were seeded with a low level (2×10^5) of viable cells in the log phase. Serial dilutions of the test peptides (0.05–10 nmol) were placed in small wells in the plate and allowed to diffuse into the agar. After 24-h incubation, the diameters of the inhibition zones around each well were measured and the lethal concentrations calculated as described previously (Hultmark et al., 1982).

RESULTS

Peptide Synthesis. The six cecropin A analogues (Figure 2) were prepared by conventional stepwise solid-phase procedures (Merrifield, 1963). The synthetic strategy chosen was based on differential acid-labile protecting groups, with the standard combination of *tert*-butyloxycarbonyl (Boc) for the α-amino function and benzyl-type derivatives for the side chains of trifunctional amino acids. In addition, tosyl (Tos) and formyl (For) were used for Arg and Trp, respectively. The 9–37 core sequence common to all six analogues was assembled

manually on a benzhydrylamine resin (Pietta & Marshall, 1970). The peptide–resin was then divided into six portions and the synthesis resumed upon each of them for every individual analogue. A double-coupling protocol was used for every synthetic cycle, consisting of a first standard DCC coupling (3 equiv, 1 h) in CH_2Cl_2, followed by a symmetrical anhydride coupling (0.5 equiv, 1 h) in DMF–CH_2Cl_2 (1:1 v/v). All residues were completely coupled after the second coupling as evidenced by the ninhydrin test (Kaiser et al., 1970), except for Val^{28}, Ile^{18}, Asn^{14}, and Gln^{13}, which required a third coupling done with preformed HOBt esters in DMF (2 equiv, overnight). Special coupling protocols for some residues were done as previously described (Andreu et al., 1983), except for the addition of Gly^{30} and Gly^{12} to Gln^{31} and Gln^{13}, respectively. For these two residues, the peptide–resin was deprotected in 4 N HCl/dioxane (Barany & Merrifield, 1980), and then, Boc-glycine was added as its *N*-methylmorpholine salt (Suzuki et al., 1975) followed by DCC. In this way, no drop of substitution was detected at this level by the quantitative ninhydrin method (Sarin et al., 1981), thus indicating that no undesirable termination by pyroglutamic acid formation had taken place, in contrast to previous results (Andreu et al., 1983). However, the substitution of the polymer decreased significantly at the level of Val^{26} (ca. 30% drop) and, in lesser degree, at other stages of the synthesis, for reasons not yet clearly understood. Thus, the initial substitution of 0.50 mmol/g of polystyrene went down to 0.125 mmol/g of polystyrene (corrected for weight gain) at the end of the synthesis. A similar decrease was observed in a previous synthesis of cecropin A (Andreu et al., 1983) and a not so pronounced one in an earlier cecropin A-(1–33) synthesis (Merrifield et al., 1982).

Cleavage and Purification. The N^{α}-Boc group was removed from the completed peptide–resins, which were further deprotected and cleaved from the resin by the new low/high HF method of Tam et al. (1983). Low HF was done with HF/dimethyl sulfide/*p*-cresol/*p*-thiocresol (25:65:5:5), at 0 °C, for 2 h. High HF was done with HF/*p*-cresol/*p*-thiocresol (95:2.5:2.5), at 0 °C, for 1 h. [Glu^2]cecropin A, which does not possess a Trp residue, was only submitted to high HF [HF/*p*-cresol (9:1), 0 °C, 1 h]. The residues were first extracted with anhydrous ether to remove the scavengers, and then the peptides were eluted from the resin with 10% HOAc. The ether layer was back-extracted with 10% HOAc, and the extract was pooled with the main peptide solution. Cleavage yields ranged from 75 to 80% on the basis of amino acid analysis of the total extract. The crude peptides were purified by a three-step procedure:

(1) Gel filtration through Sephadex G-25 in 1 M HOAc was used to separate the peptide material from low molecular weight impurities from the HF reaction.

(2) Carboxymethylcellulose (CM-52) cation-exchange chromatography, at pH 6.6, was next employed. For all six peptides, the chromatogram (Figure 3) showed the major product flanked by two other peaks, corresponding respectively to products with one less and one more positive charge, in order of elution. The product with one additional charge eluting in the last place accounted in all cases for about 15% of the main peak. It was readily identified as the result of aspartimide formation at the Asp^{17}-Gly^{18} level. Identification was done by the procedure described by Merrifield (1967), consisting of treatment with concentrated NH_4OH (1 min, room temperature) to effect aspartimide ring opening, followed by HPLC analysis of the products. Two peaks of comparable intensity were observed, in addition to some unreacted aspartimide material. Both had identical amino acid analyses.

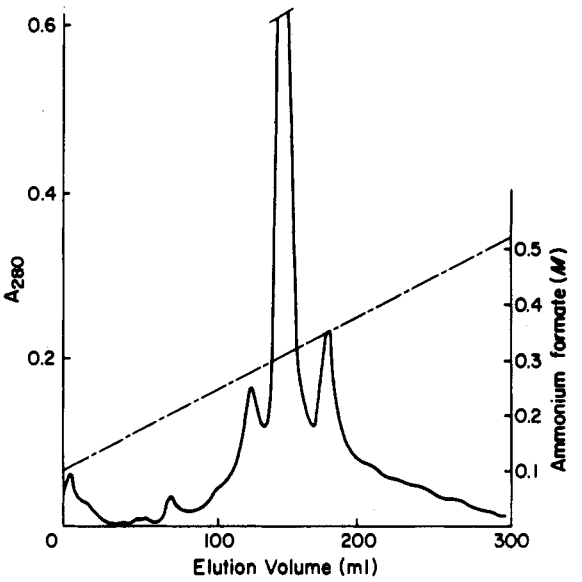

FIGURE 3: CM-52 chromatography of synthetic cecropin A analogues (shown here is [Pro^8]cecropin A). See text for conditions.

Table I: Amino Acid Analyses of Cecropin A Analogues[a]

	(2–37)	[Glu^2]	[Pro^4]	[Glu^6]	[Leu^6]	[Pro^8]
Asp	2.01 (2)	1.98 (2)	1.95 (2)	2.09 (2)	2.06 (2)	2.06 (2)
Glu	4.12 (4)	4.97 (5)	3.87 (4)	4.85 (5)	4.19 (4)	4.06 (4)
Gly	4.10 (4)	4.03 (4)	3.98 (4)	3.92 (4)	4.09 (4)	3.84 (4)
Thr	0.89	0.90	0.94	0.87	0.92	0.84
Arg	1.04	1.01	0.96	0.98	1.00	0.94
Ala	5.00 (5)	5.18 (5)	5.04 (5)	5.11 (5)	4.95 (5)	4.81 (5)
Val[b]	3.91 (4)	3.80 (4)	3.83 (4)	3.78 (4)	4.01 (4)	3.89 (4)
Phe	1.04	0.99	1.00	1.06	1.02	0.98
Ile[b]	4.77 (5)	4.80 (5)	4.63 (5)	4.72 (5)	4.74 (5)	3.94 (4)
Leu	1.05	1.03	0.00 (0)	1.01	1.99 (2)	1.00
Lys	6.03 (6)	6.58 (7)	6.62 (7)	5.94 (6)	6.20 (6)	6.59 (7)

[a] Hydrolysates from 6 N HCl, 110 °C, 24 h, unless indicated otherwise. Analysis by *o*-phthalaldehyde method. Values in parentheses are theoretical. Pro and Trp were not determined. [b] These are 72-h hydrolysis values.

One of them coeluted with the main α-aspartyl product and was therefore identified as the product of opening at the β-carbonyl; the other one had a lower retention time and was identified as the corresponding β-aspartyl peptide. The high levels of aspartimide formation detected in this synthesis were attributed to the use of benzyl ester protection for aspartic acid. The cyclohexyl esters (Tam et al., 1979), which had been used advantageously in previous syntheses, are undoubtedly to be preferred.

(3) The material eluting in the main CM-52 peak was lyophilized and submitted to reverse-phase chromatography on C_{18} silica, which afforded the corresponding cecropin A analogues in pure form. Overall purification yields were in the 30–35% range. The pure peptides were characterized by amino acid analysis (Table I), analytical HPLC (single peak in all cases, data not shown), and polyacrylamide gel electrophoresis (Figure 4). Electrophoretic mobilities of the six analogues were in total agreement with their charge differences. In the experimental conditions used (pH 4.0), differences of one amino group were more noticeable than differences of one carboxyl group.

Circular Dichroism. The CD spectra of the six synthetic cecropin A analogues in water were characteristic of random-coil, nonstructured conformations. This was also the case with cecropin A and cecropin A-(1–33). In the presence of a helix-promoting solvent such as hexafluoro-2-propanol, however, the molecules readily became structured, acquiring

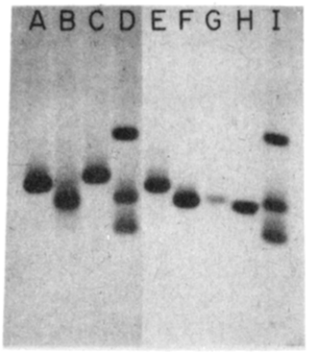

FIGURE 4: Polyacrylamide gel electrophoresis of cecropin A analogues: [lanes D and I (from top)] cecropin D, A, and B standards; (other lanes) (A) [Leu^6]cecropin A; (B) [Pro^4]cecropin A; (C) [Glu^6]cecropin A; (E) cecropin A(2–37); (F) [Pro^8]cecropin A; (G) [Glu^2]cecropin A; (H) cecropin A(3–37). All lanes contain 12 μg of peptide, except lanes D and I, which contain 6 μg of each standard.

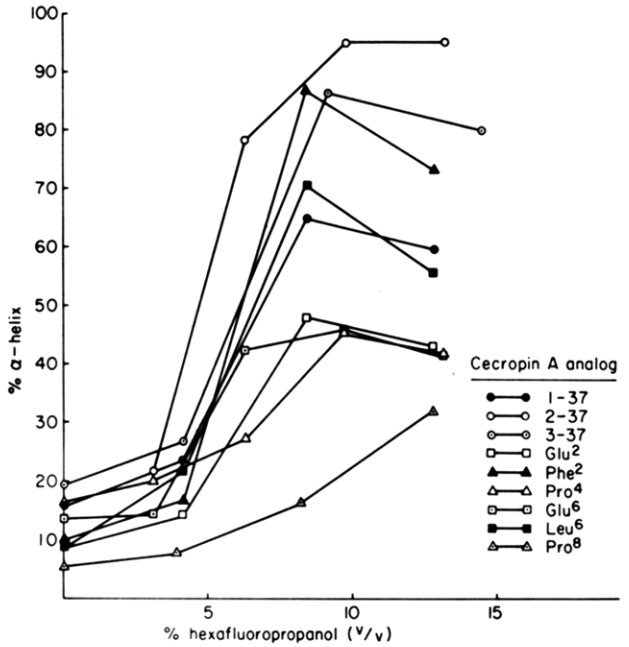

FIGURE 5: Effect of hexafluoro-2-propanol concentration on maximum helixities of cecropin A analogues calculated from ellipticities at 222 nm.

significant levels of α-helix conformation at concentrations of 15–20% of organic solvent. In Figure 5, the percentage of α-helix (Wu et al., 1981) for each analogue has been plotted as a function of the concentration of helix-promoting solvent. It can be seen that single-residue replacements had marked effects on the degree of helicity of the corresponding peptides under these conditions. Substantial decreases in helical content were observed for the [Glu^6], [Pro^4], and [Glu^2] analogues and most especially for [Pro^8]cecropin A, which had a very low α-helix content even at a high concentration of hexafluoro-2-propanol. [Leu^6]cecropin A, on the other hand, behaved somewhat like the original molecule.

Antibacterial Activity. The synthetic cecropin A analogues were tested for antibacterial activity against two Gram-negative (*Escherichia coli*, *Pseudomonas aeruginosa*) and two Gram-positive (*Bacillus megatherium*, *Micrococcus luteus*) bacteria, which have been systematically used as standards for antibacterial activity of cecropins. A classical inhibition zone assay on thin agar was used. For every bacterium, the squares of the inhibition zone diameters (d^2) were plotted against the log of the respective concentrations obtained by serial dilutions of the test peptide (Figures 6–9). The lethal

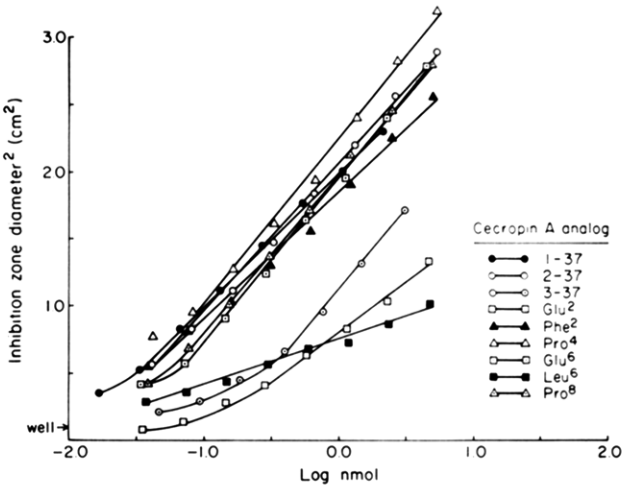

FIGURE 6: Antibacterial activity assay of cecropin A analogues against *E. coli*. Squares of inhibition zone diameters are plotted vs. log of peptide amount (in nanomoles). Lethal concentrations (Hultmark et al., 1982) can be calculated for each analogue from the slope of the corresponding line.

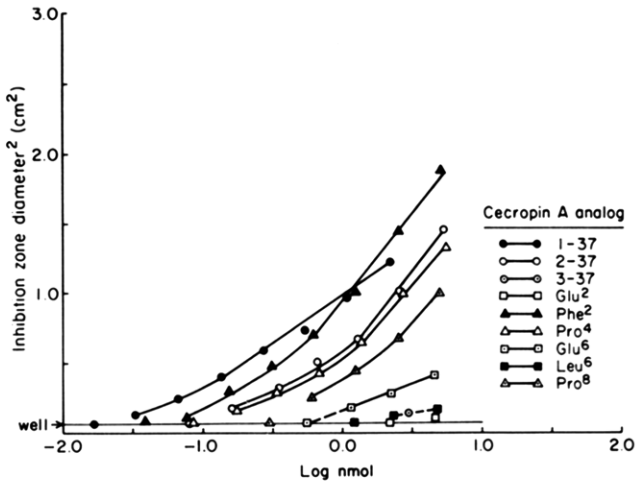

FIGURE 7: Antibacterial activity assay of cecropin A analogues against *P. aeruginosa*. Other details are as in Figure 6.

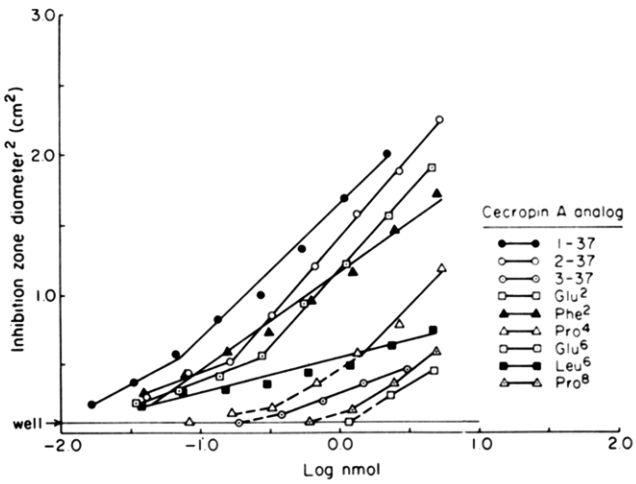

FIGURE 8: Antibacterial activity assay of cecropin A analogues against *B. megaterium*. Other details are as in Figure 6.

concentrations, summarized in Table II, were calculated for every analogue from the slopes and the intercepts of the corresponding lines, as described by Hultmark et al. (1982). Differences in lethal concentration greater than 30% were considered significant and were quite reproducible.

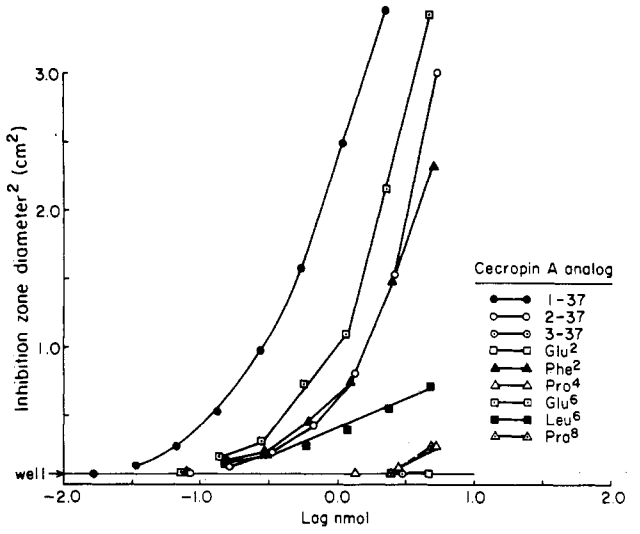

FIGURE 9: Antibacterial activity assay of cecropin A analogues against *M. luteus*. Other details are as in Figure 6.

Table II: Lethal Concentrations (μM) of Cecropin A and Synthetic Analogues

cecropin A analogue	bacterium			
	E. coli D21	*P. aeruginosa* OT 97	*B. megaterium* Bm 11	*M. luteus* M1 11
(1–37), natural	0.35	2.6	0.60	1.4
(1–37), synthetic	0.28	1.5	0.56	1.5
(2–37)	0.37	8.6	1.5	7.3
(3–37)[a]	2.6	90	13	>110
[Phe^2][a]	0.34	3.5	0.78	7.4
[Glu^2]	3.2	170	39	>170
[Pro^4]	0.36	8.1	11	87
[Leu^6]	0.56	120	0.78	7.3
[Glu^6]	0.58	34	2.2	4.7
[Pro^8]	0.50	15	31	80
(1–33)[b]	0.43	13	24	27

[a] From Andreu et al. (1983). [b] From Merrifield et al. (1982).

DISCUSSION

The purpose of this work was to explore some aspects of the structure–activity relationships in cecropin A and primarily the possibility that an amphipathic α-helix model previously proposed (Steiner, 1982; Merrifield et al., 1982) might be involved in the interaction of the molecule with the bacterial membranes. We were particularly interested in the N-terminal region, which has been shown by Chou–Fasman calculations to favor an α-helix conformation.

The evaluation of antibacterial activity was done by an inhibition zone assay in agar (Hultmark et al., 1 82). This assay is quite reproducible, and the precision of the value for lethal concentration calculated from it is estimated to be within ±30%. Therefore, the present data allow several important conclusions to be drawn.

First, the tryptophan residue in position 2 is clearly important for antibacterial activity. This residue is invariant for *Hyalophora cecropia* cecropins A, B, and D and is also found in the same position in cecropins B and D isolated from the Chinese moth *Antherea perenyi*. Its replacement by another α-helix former but nonaromatic residue like glutamic acid resulted in a sharp drop in antibacterial activity. In contrast, activity was preserved when phenylalanine was substituted for tryptophan in [Phe^2]cecropin A (Andreu et al., 1983). When deleted, as in cecropin A-(3–37) (Andreu et al., 1983), the activity against *E. coli* decreased by a factor of 10 and was almost undetectable against *P. aeruginosa* and *M. luteus*. These drastic decreases cannot be attributed to the simultaneous loss of the Lys^1 residue, since the (2–37) analogues retained substantial activity against all four test bacteria.

Another invariant residue examined was lysine at position 6. This residue is located at the center of the hydrophilic surface in the proposed 1–11 amphipathic helix model. Two analogues in this position were synthesized and tested: in one of them, lysine was replaced by a hydrophobic leucine residue that, although a good helix former, would be located in the "wrong" region of the amphipathic helix and presumably destabilize it; in the other analogue, lysine was replaced by an equally hydrophilic and helix-promoting glutamic acid residue, so that one could well assume that any variations found should be mainly attributable to the change in electric charge introduced. For [Leu^6]cecropin A, a rather anomalous dependence of inhibition vs. concentration was found (Figures 6–9), and the corresponding lethal concentrations (Table I) should therefore be considered with some caution. Both [Leu^6]cecropin A and [Glu^6]cecropin A appeared to be fully active against *E. coli*, indicating that changes in this residue at the N-terminal region had little or no effect upon the antibacterial action against this organism. Against *P. aeruginosa*, both analogues were nearly inactive, indicating an important role for Lys^6. Against the two Gram-positive organisms, the data were less conclusive.

The amphipathic helix hypothesis was further tested by the introduction of two strong helix-breaking residues in the 1–11 region of the sequence. As we have already mentioned, this section of cecropin A has, according to Chou–Fasman secondary structure predictions, the highest α-helix potential, $\langle P_\alpha \rangle = 1.17$, of all the regions. It was reasoned that a proline residue at position 4 or 8 would severely disrupt the helicity in this region. CD data showed this indeed to be the case (Figure 5). For [Pro^4]cecropin A, a 30% decrease in maximum helicity calculated from the ellipticity at 222 nm was observed. For [Pro^8]cecropin A, the effect was even more pronounced, and one could assume that practically no α-helix structure was left in the N-terminal section, any remaining helicity being due to the other half of the molecule, which these replacements did not alter. Against *P. aeruginosa*, *B. megaterium*, and *M. Luteus* the antibacterial activity was very low for both [Pro^4]cecropin A and [Pro^8]cecropin A, suggesting that the 1–11 helix is required for interaction with the membranes of these cells. In contrast, antibacterial assays for both analogues showed no substantial loss of activity against *E. coli*, which is a particularly susceptible organism that may not require participation by all portions of the peptide. The variety of responses encountered so far indicates quite clearly that no unique binding site common to all bacteria can be postulated for interaction of cecropin A. For one thing, the action of cecropin against *E. coli* seems to be based on a different kind of interaction than those of the other three bacteria tested. The absence of significant effects caused by any of the replacements introduced so far in the molecule leads us to conclude that, with the exception of the aromatic Trp^2 residue, no changes have been introduced that affect any essential features of the binding site of cecropin A to *E. coli*. On the other hand, and as previously mentioned, the molecule of cecropin A is not uniformly helical due to a disruption caused by residues Gly^{23} and Pro^{24}, which, in effect, delimit two helical sections at each side of themselves. Therefore, the possibility that an induced amphipathic α-helix at the C-terminus may be required for activity against *E. coli* remains an interesting open question. We are currently exploring it by designing new synthetic analogues in this area that should prove useful in establishing to what extent the C-terminal half of cecropin A is involved

in activity against *E. coli*. The fact that a recently described "short" cecropin A-(1–22) (DeGrado, 1983) lacking 15 residues at the carboxyl end had only about 10% of the activity of the full molecule against *E. coli* seems to reinforce this suggestion.

For the other three bacteria tested, however, the N-terminal half of cecropin A did clearly play a role in the interaction with bacteria. All replacements causing conformational changes in this region had also a marked effect on the activity of the corresponding peptide. This was especially evident in the case of the [Pro^4] and [Pro^8] analogues, for which a correlation was found between loss of helicity and lack of antibacterial activity.

There is no reason to assume that the entire length of the cecropin A molecule must be involved in the critical binding to bacterial cells. Rather, it seems that different bacteria may selectively interact with different portions of the molecule. At the same time, some permanent features such as the aromatic residue in position 2 appear to be a more general requirement. Incidentally, these results illustrate a way in which natural selection could operate in the development of insect immunity. From an evolutionary perspective, one could view cecropin A as a compromise or equilibrium response to several selection pressures: those in the quest for an effective antibacterial structure capable of acting upon a variety of Gram-positive and Gram-negative bacteria and those of self-preservation, calling for a molecule specific enough to be active on bacteria but nontoxic for the insect itself. This is in contrast with mellitin and other membrane-active peptides, which destroy eucaryotic cells as well.

Registry No. Cecropin A, 80451-04-3; (2–37)cecropin A, 94992-98-0; [Glu^2]cecropin A, 94992-97-9; [Pro^4]cecropin A, 94993-01-8; [Glu^6]cecropin A, 94992-99-1; [Leu^6]cecropin A, 94993-02-9; [Pro^8]cecropin A, 94993-00-7; [Phe^2]cecropin A, 88845-02-7; (3–37)cecropin A, 88845-01-6; (1–33)cecropin A, 81541-05-1.

References

Andreu, D., Merrifield, R. B., Steiner, H., & Boman, H. G. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 6475–6479.

Assman, G., & Brewer, H. B., Jr. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 1534–1538.

Barany, G., & Merrifield, R. B. (1980) in *The Peptides* (Gross, E., & Meienhofer, J., Eds.) pp 1–284, Academic Press, New York.

Boman, H. G., & Steiner, H. (1981) *Curr. Top. Microbiol. Immunol. 94*, 75–91.

Crestfield, A. M., Moore, S., & Stein, W. H. (1963) *J. Biol. Chem. 238*, 622.

DeGrado, W. F. (1983) in *Peptides: Structure and Function* (Hruby, V., & Rich, D. H., Eds.) pp 195–198, Pierce Chemical Co., Rockford, IL.

Hultmark, D., Engström, Å, Bennich, H., Kapur, R., & Boman, H. G. (1982) *Eur. J. Biochem. 127*, 207–217.

Kaiser, E. T., & Kezdy, F. J. (1984) *Science (Washington, D.C.) 223*, 249–255.

Kaiser, E., Colescott, R. L., Bossinger, C. D., & Cook, P. I. (1970) *Anal. Biochem. 34*, 595–598.

Merrifield, R. B. (1963) *J. Am. Chem. Soc. 85*, 2149–2154.

Merrifield, R. B. (1967) *Recent Prog. Horm. Res. 23*, 451–482.

Merrifield, R. B., Vizioli, L. D., & Boman, H. G. (1982) *Biochemistry 21*, 5020–5031.

Pietta, P. G., & Marshall, G. R. (1970) *J. Chem. Soc. D.*, 650–651.

Sarin, V. K., Kent, S. B. H., Tam, J. P., & Merrifield, R. B. (1981) *Anal. Biochem. 117*, 147–157.

Segrest, J. P., Jackson, R. L., Morrisett, J. P., & Gotto, A. M. (1974) *FEBS Lett. 38*, 247–253.

Steiner, H. (1982) *FEBS Lett. 137*, 283–287.

Suzuki, K., Nitta, K., & Endo, N. (1975) *Chem. Pharm. Bull. 23*, 222–224.

Tam, J. P., Wong, T. W., Riemen, M. W., Tjoeng, F. S., & Merrifield, R. B. (1979) *Tetrahedron Lett.*, 4033–4036.

Tam, J. P., Heath, W. F., & Merrifield, R. B. (1983) *J. Am. Chem. Soc. 105*, 6442–6455.

Westall, F. C., Scotchler, J., & Robinson, A. B. (1972) *J. Org. Chem. 37*, 3363–3364.

Wu, C. C., Ikeda, K., & Yang, J. T. (1981) *Biochemistry 20*, 566–570.