Butler, P. J. G., & Thomas, J. O. (1980) *J. Mol. Biol. 140*, 505–529.
Campbell, A. M., Cotter, R. I., & Pardon, J. F. (1978) *Nucleic Acids Res. 5*, 1571–1580.
Crothers, D. M., Dattagupta, N., Hogan, M., Klevan, L., & Lee, K. S. (1978) *Biochemistry 17*, 4525–4533.
Finch, J. T., & Klug, A. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 1897–1901.
Finch, J. T., Brown, R. S., Rhodes, D., Richmond, T., Rushton, B., Lutter, L. C., & Klug, A. (1981) *J. Mol. Biol. 145*, 757–769.
Fulmer, A. W., & Bloomfield, V. A. (1982) *Biochemistry 21*, 985–992.
Houssier, C., Bontemps, J., Edwards-Alt, X., & Fredericq, E. (1977) *Ann. N.Y. Acad. Sci. 303*, 170–189.
Lee, K. S. (1980) Thesis, Yale University.
Lee, K. S., & Crothers, D. M. (1982) *Biopolymers 21*, 101–116.
Lee, K. S., Mandelkern, M., & Crothers, D. M. (1981) *Biochemistry 20*, 1435–1438.
McGhee, J. D., Rau, D. C., Charney, E., & Felsenfield, G. (1980) *Cell (Cambridge, Mass.) 22*, 87–96.
Rattner, J. B., & Hamkalo, B. A. (1978) *Chromosoma 69*, 363–379.
Rill, R., & Van Holde, K. E. (1974) *J. Mol. Biol. 83*, 459–471.
Ris, H., & Kubai, D. F. (1970) *Annu. Rev. Genet. 4*, 263–294.
Sperling, L., & Klug, A. (1977) *J. Mol. Biol. 112*, 253–263.
Suau, P., Bradbury, E. M., & Baldwin, J. P. (1979) *Eur. J. Biochem. 97*, 593–602.
Thoma, F., Koller, T., & Klug, A. (1979) *J. Cell Biol. 83*, 403–427.
Thomas, J. O., & Butler, P. J. G. (1980) *J. Mol. Biol. 144*, 89–93.
Tjerneld, J., & Norden, B. (1982) *Biopolymers 21*, 343–358.
Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406–4412.

Synthesis of the Antibacterial Peptide Cecropin A(1–33)†

R. B. Merrifield,* L. D. Vizioli, and H. G. Boman

ABSTRACT: Cecropin A(1–33) was synthesized by an improved stepwise solid-phase method. The synthesis was designed to give high coupling yields and minimal amounts of byproducts. All coupling steps were monitored for completion by a new ninhydrin procedure, and the fully protected peptide-resin was analyzed for deletion peptides by the solid-phase Edman preview technique. Both methods indicated that the average coupling yield was >99.8%. The unpurified peptide mixture resulting from HF cleavage and extraction into 10% acetic acid was analyzed by reverse-phase high-pressure liquid chromatography, and 93% of the total product was shown to be the desired [Trp(For)^2]cecropin A(1–33), indicating an average yield per synthetic cycle of 99.8%. Removal of the formyl group at pH 9, followed by ion-exchange chromatography, gave the purified product. Cecropin A(1–33) showed antibacterial activity against both Gram-positive and Gram-negative bacteria. Against *Escherichia coli*, the activity was only slightly lower than that of the natural 37-residue cecropin A when tested over a 100-fold concentration range; the minimum inhibitory concentration was approximately 1 μM. The formyl derivative was somewhat less effective in killing *E. coli* than the free 1–33 peptide. The antibacterial activity was discussed in terms of an amphipathic α-helix structure and the binding of the peptide to bacterial membranes.

The cecropins are a newly discovered class of antibacterial peptides produced by the humoral immune response of certain insects (Hultmark et al., 1980; Boman & Hultmark, 1981; Boman & Steiner, 1981). Cecropins and about 10 other immune proteins are induced in the hemolymph of the pupae of the giant silk moth *Hylophora cecropia* following injection of live bacteria. The first cecropins to be purified to homogeneity and for which tentative primary structures are available are the A and B forms (Steiner et al., 1981). They each contain 37 amino acid residues, with a basic N-terminal region and a hydrophobic C-terminal region ending with a blocked carboxyl groups. Cecropins A and B are strongly homologous but differ significantly in structure and function from other known basic peptides such as melittin.

The cecropins are antibacterial against a variety of Gram-negative bacteria and therefore differ from lysozyme, which is effective only against certain Gram-positive bacteria. The range of susceptible pathogens is broader than that for melittin, and in addition, the latter is lytic for Chang liver cells whereas cecropins have no effect on these cells or on sheep erythrocytes or insect cells in tissue culture (Steiner et al., 1981).

A synthetic program on the cecropins has been undertaken with the objectives of confirming their structure, providing sufficient material to enable more extensive studies on their mode of action, and examining the role of their very interesting structures on their bactericidal activity. This paper describes in detail the solid-phase synthesis of cecropin A(1–33), which at the initiation of the work was believed to represent the complete sequence of the molecule. The structure of the synthetic protected peptide-resin I is

Boc-Lys(ClZ)-Trp(For)-Lys(ClZ)-Leu-Phe-Lys(ClZ)-Lys(ClZ)-Ile-Glu(*O*Bzl)-Lys(ClZ)-Val-Gly-Gln-Asn-Ile-Arg(Tos)-Asp(*O*cHex)-Gly-Ile-Ile-Lys(ClZ)-Ala-Gly-Pro-Ala-Val-Ala-Val-Val-Gly-Gln-Ala-Thr(Bzl)–OCH_2–C_6H_4–$CH_2CONHCH_2$–C_6H_4–resin

I

The synthesis followed the stepwise solid-phase strategy (Merrifield, 1963), with the acid-labile *tert*-butyloxycarbonyl (Boc)[1] group for temporary N^{α} protection and more acid-stable

† From the Rockefeller University, New York, New York 10021 (R.B.M. and L.D.V.), and the Department of Microbiology, University of Stockholm, S-10691 Stockholm, Sweden (H.G.B.). *Received March 17, 1982.* The work was supported by Grant AM01260 from the U.S. Public Health Service and by a grant from the Swedish Natural Science Foundation to H.G.B. (BU2453-108).

0006-2960/82/0421-5020$01.25/0

groups for protection of the side chains. N^{ϵ}-(2-Chlorobenzyloxycarbonyl)lysine [Lys(ClZ)] (Erickson & Merrifield, 1972) was used because it is much more stable than the benzyloxycarbonyl derivative to the acid deprotection steps and avoids side-chain branching. The β-cyclohexyl (cHex) ester of (*tert*-butyloxycarbonyl)aspartic acid (Tam et al., 1979b) was used because it is also more acid stable and it minimizes aspartimide formation. Trytptophan was protected with the N^{i}-formyl (For) group (Previero et al., 1967) to prevent tert-butylation or oxidation of the indole during the acid steps (Ohno et al., 1972; Yamashiro & Li, 1973).

The key to the synthesis was the use of [[[4-(oxymethyl)-phenyl]acetamido]methyl]copoly(styrene–divinylbenzene) resin (–OCH_2-Pam-resin) as the solid support (Mitchell et al., 1976). The ester linkage between the peptide and the resin support has enhanced acid stability compared with that of the usual benzyl ester linkage and reduces the acidolytic losses of peptide chains during the synthesis. It also prevents the termination of growing peptide chains by trifluoroacetylation (Kent et al., 1979).

Special attention was given to attaining as complete a coupling reaction as possible at each cycle of the synthesis. For that purpose, the first coupling was with the preformed symmetric anhydride (Hagenmaier & Frank, 1972) in dichloromethane, and the second was with the preformed hydroxybenzotriazole ester (König & Geiger, 1970) in dimethylformamide. The progress of the synthesis was monitored with a new quantitative ninhydrin procedure (Sarin et al., 1981). The completed peptide, while still attached to the solid support, was evaluated by solid-phase sequencing techniques (Laursen, 1971) for correctness of sequence and, especially, for any indication of deletions by preview analysis (Niall et al., 1972). This procedure was possible only because the bond between the peptide and the Pam-resin was sufficiently stable to acid. Purification of the peptide was by ion-exchange chromatography, and final analysis for homogeneity was by reverse-phase high-pressure liquid chromatography and gel electrophoresis. The homogeneous cecropin A(1–33) was found to possess antibacterial activity against one Gram-positive and three Gram-negative bacteria.

Experimental Procedures

Materials. Commercial protected amino acids were obtained from Peninsula Laboratories, San Carlos, CA. The purity was assessed by melting points and thin-layer chromatography. Other reagents were trifluoroacetic acid (Halocarbon Products), HF (Matheson), diisopropylethylamine and pyridine (Aldrich), both distilled over ninhydrin (Pierce), dicyclohexylcarbodiimide (Schwarz/Mann), 1-hydroxybenzotriazole (Aldrich), recrystallized, dichloromethane (Eastman), distilled from Na_2CO_3, and acetonitrile, HPLC grade (Burdick and Jackson).

General Methods. Hydrolysis of free peptides was with 6 N HCl in evacuated, sealed tubes at 110 °C, 24 h, according to Crestfield et al. (1963). Peptide-resins were hydrolyzed in 12 N HCl/phenol/HOAc (2:1:1) at 110 °C, 24 h, as described by Gutte & Merrifield (1971), and in 4 N methanesulfonic acid, 110 °C, 24 h, according to Simpson et al. (1976). HF reactions were carried out in a Diaflon HF apparatus (Toho Co., Osaka, Japan).

Preparative carboxymethyl-Sepharose columns were eluted with a linear gradient of ammonium acetate, 0.1–0.5 M (150 mL each), pH 6.6, at 6 mL/h. The effluent was monitored at 280 nm on an Altex Model 153 flow-through spectrophotometer.

Analytical high-pressure liquid chromatography of the peptides was on a thermostated (31 °C), reverse-phase μBondapak C-18 column (4 × 300 mm) in a Waters Associates instrument fitted with a Schoeffel variable-wavelength UV photometer and an automatic Wisp injector. The chromatograms were recorded on a Hewlett-Packard 3380 A integrator (1 mV full scale). Solution A contained 900 mL of H_2O, 100 mL of CH_3CN, and 1 mL of 85% H_3PO_4; solution B contained 200 mL of H_2O, 800 mL of CH_3CN, and 1 mL of 85% H_3PO_4. The linear gradient was from 10 to 50% solution B into solution A in 25 min, 2 mL/min.

Polyacrylamide Gel Electrophoresis with Bioassay. Electrophoretic characterization of antibacterial substances was according to Hultmark et al. (1980) on 15% polyacrylamide gels in acetate buffer, pH 4, with the acrylamide/bis(acrylamide) ratio 75:1. The gel was run at 200 V, for about 3.5 h. The tracker dye, methyl green, left the gel 1 h before the end of the run. A small amount of antibacterial impurity in the dye moved with approximately 40% of the mobility of methyl green itself. After the separation, the gels were incubated for 1 h in a rich medium containing 0.2 M sodium phosphate, pH 7.4, and streptomycin, 100 μg/mL. The gel (8 × 8 cm) was then overlayed with about 6 mL of melted soft agar (0.6%) containing the indicator bacteria (4×10^4 viable cells/mL), either *Escherichia coli* D31 or *Bacillus megaterium* Bm11, both resistant to streptomycin. On top of this was poured another layer of agar without bacteria, and the gel was incubated at 37 °C overnight. For purity tests, the gels were fixed in 20% 5-sulfosalicylic acid and stained with Coomassie brilliant blue R in 7% acetic acid.

$NaDodSO_4$ Gel Electrophoresis. Sodium dodecyl sulfate electrophoresis was carried out in 15% polyacrylamide gels [acrylamide/bis(acrylamide) ratio 40:1] poured in 0.1 M sodium phosphate, pH 7.2, with 6 M urea and 0.1% $NaDodSO_4$; the running buffer was 0.1 M sodium phosphate, pH 7.2, and 0.1% $NaDodSO_4$. The peptides were treated for 5 min at 100 °C in 0.1 M phosphate, with 1% $NaDodSO_4$, 1% mercaptoethanol, and 7 M urea. Electrophoresis was at 55 V for 30 h. The gels were fixed in 10% trichloroacetic acid/30% MeOH and stained with 0.1% Coomassie brilliant blue R in 10% acetic acid, 25% 2-propanol, and 0.1% $Cu(NO_3)_2$.

Assay of Antibacterial Activity. The assay is a variation of the classical method used for antibiotics. The agar plates (8.5 cm in diameter) were prepared with 6 mL of rich medium containing streptomycin (100 μg/mL) and about 8×10^4 viable cells of a test organism resistant to streptomycin. For assurance of an equal thickness of medium, the plates were poured on a water level adjusted table. Wells of 3-mm diameter were punched in the plates. The test material was dissolved in water and serially diluted in Eppendorf tubes, and 3 μL was applied to each well. The diameters of the inhibition zones around the wells were measured after overnight incubation at 37 °C.

Minimum inhibitory concentration (mic) was determined in 50-μL cultures after 20-h incubation at 37 °C of growing log-phase cells in a rich medium containing streptomycin (100 μg/mL). Cecropins were added in 2-fold dilutions. The inoculum concentration was 10^5 viable cells/mL.

[1] Abbreviations: *tert*-butyloxycarbonyl; Bzl, benzyl; ClZ, 2-chlorobenzyloxycarbonyl; cHex, cyclohexyl; CM, carboxymethyl; DCC, dicyclohexylcarbodiimide; DIEA, diisopropylethylamine; DMF, dimethylformamide; For, formyl; HOBt, 1-hydroxybenzotriazole; HPLC, high-pressure liquid chromatography; $NaDodSO_4$, sodium dodecyl sulfate; mic, minimum inhibitory concentration; Pam, (phenylacetamido)-methyl; PTH, phenylthiohydantoin; Tos, *p*-toluenesulfonyl; PITC, phenyl isothiocyanate.

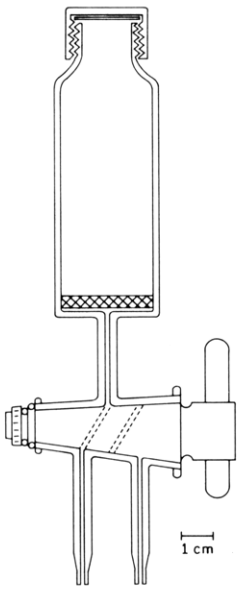

FIGURE 1: Reaction vessel for solid-phase peptide synthesis.

Peptide Sequencing and Preview Analysis. Peptide sequencing was with a Sequemat Mini 15 solid-phase sequencer with a standard program, with a few milligrams of peptide-resin (~0.5–1 μmol). Conversion of the thiazolinone derivatives to the phenylthiohydantoins was performed manually with 2 N HCl in methanol at 65 °C for 10 min. After evaporation of the reagent under a stream of N_2, the residue was taken up in 400 μL of methanol containing 0.037 mg/mL of PTH-Tyr as an internal standard. For preview analysis 40 μL was injected. The PTH-amino acid derivatives were analyzed by HPLC on a 4 × 300 mm μBondapak C-18 column by a modification of the general procedure of Bhown et al. (1978). Buffer A contained 900 mL of H_2O, 100 mL of methanol, 2.5 mL of HOAc, and 50 μL of acetone, adjusted to pH 4.1 with NaOH; buffer B contained 100 mL of H_2O, 900 mL of methanol, and 0.25 mL of HOAc. Both solutions were deaerated, and a linear gradient of 5–85% buffer B was run over a 40-min period at 2 mL/min.

Reaction Vessel. Solid-phase synthesis was performed manually on a shaker similar to one described previously (Merrifield et al., 1966). A newly designed reaction vessel (Figure 1) was used. It consists of a 60 mL capacity glass cylinder (30 × 110 mm) made from a screw-capped Pyrex culture tube (screw cap fitted with a Teflon interface, Scientific Glass Inc.) (Gisin & Merrifield, 1972), fitted at the lower end with a coarse porosity fritted disc (Corning) and a two-way 1.5-mm stopcock. The Teflon inlet and outlet lines (0.076-in. i.d.) pass through the axis of the shaker arm. The shaker inverts the vessel through a 180° arc at a rate of 25 cycles/min. The resin containing the first amino acid residue is placed in the vessel, the top rim is carefully freed of solid particles, and the cap is closed. With the stopcock in the "drain" position the vessel is evacuated through a trap with an aspirator. The stopcock is turned to "fill", and the solvent is drawn in from a reservoir by the vacuum in the vessel. The stopcock is closed and the shaking commenced. After the prescribed time, the cycle of filtration, filling, and mixing is repeated. This simple, convenient apparatus has two important advantages over our previous manual vessel or the available commercial systems: (a) it avoids drawing air through the peptide during filtration and the accompanying evaporation, cooling, and condensation of moisture on the resin, and (b) it avoids the need to open the vessel each time solvents and reagents are added, while still providing access to samples as required.

Resin Support. The support for the synthesis was based on copoly(styrene–1% divinylbenzene) resin, 200–400 mesh beads (Bio-beads), obtained from Bio-Rad Laboratories. The beads (50.0 g) were thoroughly washed in a three-neck round-bottom flask by slow stirring with an overhead paddle stirrer at 70 °C with 10 volumes of toluene, dimethylformamide, dioxane, and 2 N aqueous HCl in dioxane (1:1), 3 times each. Solvents were removed by suction through a filter stick. The beads were then washed on a funnel with dioxane and methanol and dried in vacuo at room temperature, yield 49.8 g.

The beads were sized by suspending 20 g in a graduated cylinder in 1 L of methanol and decanting from a small amount of dense material. The suspension was allowed to stand until approximately 95% had settled before decanting the supernate containing a suspension of the fine particles. This was repeated 3 times. The sizing was continued by suspending the resin in 1 L of dichloromethane in a separatory funnel, and after about 98% had floated to the top, the solvent containing a small amount of fines was withdrawn. This was repeated 3 times. The product was dried in vacuo at room temperature, yield 16.7 g. The average diameter, measured under a microscope, was 71 ± 11 μm. A 3.7-g fraction of smaller beads, 45 ● 6 μm average diameter, was also recovered.

Washed and sized resin beads (15 g) were converted to aminomethyl-resin according to Mitchell et al. (1976, 1978). The ratio of IR peaks of the intermediary phthalimidomethyl-resin at 1720/1601 cm^{-1} was 1.09, indicating a substitution of 0.19 mmol/g. The phthaloyl group was then removed by hydrazinolysis, yield 11.3 g. The phthaloyl carbonyls at 1725 and 1780 cm^{-1} were absent.

Boc-Thr(Bzl)-[4-(oxymethyl)phenyl]acetic Acid. This compound was prepared by the general methods of Tam et al. (1979a) from Boc-Thr(Bzl)-OH (3.7 g, 12 mmol), powdered $KF{\cdot}2H_2O$ (2.50 g, 26.4 mmol), and [4-(bromomethyl)phenyl]acetic acid phenacyl ester (2.08 g, 6 mmol). The product was isolated as the cyclohexylamine salt and recrystallized by dissolution in 20 mL of CH_2Cl_2 and addition of 60 mL of petroleum ether: yield 2.88 g (51%); mp 147.5–149 °C. Anal. Calcd for $C_{31}H_{44}N_2O_7$ (556.77): C, 66.87; H, 7.98; N, 5.03. Found: C, 66.90; H, 7.95; N, 4.95. Boc-Thr(Bzl)–$OCH_2C_6H_4CH_2COO^{-+}NH_3C_6H_{11}$ was quantitatively converted to the acid by shaking a solution in CH_2Cl_2 with 0.5 M $KHSO_4$ (Spangenberg et al., 1971).

Synthetic Protocols. Several procedures were used, depending on the particular amino acids involved in the coupling step. The following are based on 2.2 g of starting resin.

(A) Procedure for Gly, Ala, Val, Leu, Ile, Pro, Phe, Glu(*O*Bzl), Lys(ClZ), and Trp(For): (1) CH_2Cl_2, 30 mL, 3 × 1 min; (2) 50% F_3CCOOH/CH_2Cl_2, 30 mL, 3 × 1 min; (3) 50% F_3CCOOH/CH_2Cl_2, 30 mL, 20 min; (4) CH_2Cl_2, 30 mL, 6 × 1 min; (5) 5% DIEA/CH_2Cl_2, 30 mL, 2 × 2 min; (6) CH_2Cl_2, 30 mL, 6 × 1 min; (7) 5-mg sample for ninhydrin; (8) protected amino acid, 8 equiv in 10 mL of CH_2Cl_2, 0 °C, add DCC, 4 equiv in 3 mL of CH_2Cl_2, after 10 min, 0 °C, filter, add to reaction vessel, followed by a 10-mL CH_2Cl_2 rinse (final concentration of symmetric anhydride ~0.05 M), shake 2 h, room temperature; (9) CH_2Cl_2, 30 mL, 4 × 2 min; (10) 5% DIEA/CH_2Cl_2, 30 mL, 2 min; (11) CH_2Cl_2, 30 mL, 4 × 1 min; (12) 5-mg sample for ninhydrin; (13) DMF, 30 mL, 2 × 2 min; (14) HOBt, 4 equiv in 7 mL of DMF, 0 °C, add DCC, 4 equiv in 3 mL of CH_2Cl_2, add protected amino acid, 4 equiv in 6 mL of DMF, rinse with 4 mL of DMF, 10 min, 0 °C, add to reaction vessel, rinse with 4 mL of DMF, shake

2 h, room temperature; (15) DMF, 30 mL, 2 × 2 min; (16) CH_2Cl_2, 30 mL, 4 × 1 min; (17) 5% DIEA/CH_2Cl_2, 30 mL, 2 min; (18) CH_2Cl_2, 30 mL, 3 × 1 min; (19) 5-mg sample for ninhydrin.

(B) Procedure for Asn and Gln: (1) to (7) same as in (A); (8) DMF/CH_2Cl_2 (1:2), 30 mL, 2 × 2 min; (9) HOBt, 4 equiv in 7 mL of DMF/CH_2Cl_2 (1:1), 0 °C, add DCC, 4 equiv in 3 mL of CH_2Cl_2, add protected amino acid, 4 equiv in 6 mL of DMF/CH_2Cl_2 (1:2), rinse with 4 mL of DMF/CH_2Cl_2 (1:2), 10 min, 0 °C, add to reaction vessel, rinse with 4 mL of DMF/CH_2Cl_2 (1:2), shake 2 h, room temperature; (10) DMF/CH_2Cl_2 (1:2), 30 mL, 2 × 2 min; (11) to (21) same as (9) to (19) in (A).

(C) Procedure for Arg(Tos): (1) to (7) same as in (A); (8) protected amino acid, 4 equiv in 10 mL of CH_2Cl_2, add to reaction vessel, after 5 min add DCC, 4 equiv in 3 mL of CH_2Cl_2, rinse with 10 mL of CH_2Cl_2, shake 2 h, room temperature; (9) to (19) same as in (A).

Results

Assembly of Protected Peptide-Resin with the Sequence of Cecropin A(1–33). Aminomethyl-resin (2.2 g, 418 μmol) was placed in the reaction vessel and allowed to swell by shaking in 30 mL of CH_2Cl_2 for 1 h. It was then coupled in CH_2Cl_2 with Boc-Thr(Bzl)–$OCH_2C_6H_4CH_2COOH$ (571 mg, 1.25 mmol) by addition of dicyclohexylcarbodiimide (DCC) (258 mg, 1.25 mmol). Any unreacted amino groups were blocked by acetylation with a 1:1 (v/v) mixture of acetic anhydride and pyridine. A quantitative ninhydrin analysis (Sarin et al., 1981) on 10 mg of the resin showed that less than 0.06 μmol of free amine remained (<0.015% of the original aminomethyl groups). After removal of the Boc group by TFA, 420 μmol of amine was measured by the ninhydrin reaction with an effective extinction coefficient $\epsilon' = 1.56 \times 10^4$ M^{-1} cm^{-1}.

All subsequent amino acids were added with a double coupling protocol, which varied depending on the particular residue. For all residues except Boc-Arg(Tos), Boc-Asn, Boc-Gln, and the residue following Gln the first coupling was with the preformed symmetric anhydride in CH_2Cl_2 and the second was with the preformed hydroxybenzotriazole ester in DMF. For Boc-Arg(Tos) the first coupling was with DCC in CH_2Cl_2 to avoid lactam formation, and the second was with the HOBt ester in DMF. For the amides, Boc-Asn and Boc-Gln, the HOBt ester was used for both couplings to minimize nitrile formation (Mojsov et al., 1980). For coupling Boc-Gly to Gln the first coupling was with the preformed symmetric anhydride in DMF to minimize pyrrolidonecarboxylic acid formation (DiMarchi et al., 1982). A third coupling was introduced at cycles 6, 8, 26, 28, 29, 30, and 33 with the symmetric anhydride in DMF. After removal of approximately 350 mg of analytical samples, the total weight of the fully protected peptide-resin recovered at the end of the synthesis was 2.44 g.

Ninhydrin Monitoring of the Synthesis. A sample of peptide-resin was removed for ninhydrin analysis before every coupling reaction to determine the number of growing chains and after each reaction to determine the extent of the coupling and to decide whether or not a third coupling was needed. Part of the data are shown in Table I. After the first coupling of the first three residues the percent of uncoupled amine was below 0.1% of the total growing chains, which is near the lower limit of this ninhydrin procedure. For the remainder of the synthesis, from Gly^{30} through $Lys(ClZ)^1$, the detectable level of free amine after one coupling averaged 1.9 μmol/g (range 0.5–5.8 μmol/g). This was reduced to an average of 0.55 μmol/g after a second, or in some cases a third, coupling. For the early part of the synthesis it could be estimated by this method that the couplings were greater than 99.5% complete, and for the later part the value was approximately 99%. The deletion data from sequencing experiments to be described indicate that the reactions are more complete than this (~99.9%), and part of the ninhydrin color is attributed to nonspecific background reactions. Proline has been shown, as expected, to give a resin-bound yellow color following the ninhydrin reaction but no absorbance at 570 nm in solution. Therefore, the monitoring data for deprotected Pro^{24} (0.42 μmol/g) and after coupling Boc-Gly^{23} to Pro^{24} (0.49 and 0.31 μmol/g) suggest a general background level of approximately 0.4 μmol/g for the ninhydrin reaction under these conditions (5 min, 100 °C). The value rises with longer heating. A correction of 0.4 μmol/g gives an average coupling completion of 99.8% for this synthesis. The ninhydrin data on the fully deprotected peptide chain at each step showed a rather rapid drop in growing chains following Gln^{31} (to 0.115 mmol/g of resin) and only a slow decrease for the remainder of the chain assembly to a final value of 73 μmol/g of resin.

Sequence Analysis of the Synthesis. Fully protected cecropin A(1–33) peptide-resin (7.4 mg, 459 nmol) was mixed with glass beads and placed in the column of a Sequemat Mini 15 solid-phase peptide sequencer. The Boc group was removed by a shortened program, and then 33 steps of the standard sequencing program were carried out automatically. The thiazolinone derivatives of the amino acids were collected and converted by a manual procedure to the PTH derivatives.

Table I: Ninhydrin Monitoring of Synthesis of Cecropin A(1–33)[a]

synthesis step no.	protected residue	ninhydrin monitoring (μmol/g) after coupling no. 1	2	3
1	Thr(Bzl)	0.06		
2	Ala	0.04		
3	Gln	0.10		
4	Gly	0.96	0.73	
5	Val	0.76	0.20	
6	Val	2.28	0.47	0.50
7	Ala	1.04	0.24	
8	Val	5.84	1.28	0.41
9	Ala	0.93	0.58	
10	Pro[b]		0.42	
11	Gly	0.49[c]	0.31[c]	
12	Ala	0.48	0.10	
13	Lys(ClZ)	0.56	0.32	
14	Ile	0.59	0.43	
15	Ile	1.68	0.38	
16	Gly	0.40	0.52	
17	Asp(*O*cHex)	2.28	0.54	
18	Arg(Tos)	1.17	0.38	
19	Ile	2.85	0.69	
20	Asn	1.77	0.85	
21	Gln	2.00	0.69	
22	Gly	1.60	0.92	
23	Val	1.20	0.90	
24	Lys(ClZ)	2.20	0.89	
25	Glu(*O*Bzl)	2.30	0.40	
26	Ile	3.78	1.70	0.77
27	Lys(ClZ)	1.46	0.84	
28	Lys(ClZ)	2.98	1.14	0.64
29	Phe	4.30	1.02	0.70
30	Leu	1.99	0.98	0.25
31	Lys(ClZ)	1.91	0.71	
32	Trp(For)		0.44	
33	Lys(ClZ)	2.14	1.05	0.79

[a] An effective extinction coefficient of 1.56×10^4 M^{-1} cm^{-1} was used for all residues except for Gly (1.20×10^4) and Thr(Bzl) (1.30×10^4). [b] Proline does not give a blue color. [c] Blue color is not due to uncoupled Pro.

Table II: Repetitive Yields in Sequencing of Cecropin A(1-33)-resin

residue	peak area	interval	repetitive yield (av %/step)
Lys(ClZ)[1]	1369	1-7	94.8
Lys(ClZ)[7]	991	7-10	96.7
Lys(ClZ)[10]	897	10-21	95.3
Lys(ClZ)[21]	530	1-21	95.4
Ala[22]	631	22-25	94.4
Ala[25]	530	25-27	98.1
Ala[27]	510	27-32	94.2
Ala[32]	378	22-32	95.0
Gly[23]	479	23-30	96.7
Gly[30]	380		
Ile[8]	1048	8-15	96.3
Ile[15]	803	15-19	95.2
Ile[19]	659	8-19	95.9
Val[11]	989	11-26	96.3
Val[26]	564	26-28	95.1
Val[28]	510	28-29	95.1
Val[29]	485	11-29	96.1
		av	95.8

These were dissolved in methanol containing PTH-Tyr as an internal standard and analyzed on a Waters μBondapak C-18 column as described under Experimental Procedures. The peaks at each cycle representing (1) the main amino acid residue, (2) the carry-over of the previous residue, (3) the preview of the next residue, and (4) the background level of the amino acid two or three residues beyond, as well as the internal Tyr standard, were all integrated and recorded. The identification of the PTH-amino acids, including the various protected residues, was based on the position of standards. Lys(ClZ), Arg(Tos), and Trp(For) were stable under the conditions used for the analysis, and the PTH derivatives of the corresponding free amino acids were not detected. Varying amounts of the PTH-methyl esters were produced from Asn (33%), Gln (42%), Asp(*O*cHex) (24%), and Glu(*O*Bzl) (18%).

From these data the sequence of the synthetic chain could be unequivocally confirmed to be I, and there was no evidence for the presence of any other sequence in the product. The repetitive sequencing yields could be calculated at several positions in the chain by comparison with the peak area of the same residue at a previous position. The values ranged from 95 to 98% per step, average of 95.8% (Table II). The repetitive yields were essentially the same regardless of the position in the chain, and there were no significant differences between the yields of the various amino acid residues examined.

The most important information from the sequencing experiment is the level of preview in each cycle and hence the level of deletion peptides to be expected in the synthesis (Niall et al., 1972). The values for the total, cumulative preview of the next residue in the sequence are shown in Table III for each cycle where the determination could be made. (Because of repeat residues and carry-over, preview analysis cannot be made at all cycles.) This number is given as the percent of the total value found for that residue in the next cycle. The preview gradually increased from 0.3% at cycle 1 to 3.3% at cycle 31. Since this number is not expected to decrease at any point, the values at residues such as Asn[14] or Gly[23], which appear to be too high, are attributed to experimental error and are an indication of the limits of precision that were attained. The difference between successive values is a measure of the deletion of the residue at that cycle, but because of the low numbers involved, the precision at any one step is not high.

Table III: Preview Analysis of Synthetic Cecropin A(1-33)-resin

residue no.	protected residue	cumulative preview[a] (% total chains)	av preview/ step (%)
1	Lys(ClZ)	0.27	0.27
2	Trp(For)		
3	Lys(ClZ)	0.24	0.08
4	Leu	1.1	0.27
5	Phe	1.9	0.38
6	Lys(ClZ)		
7	Lys(ClZ)	2.9	0.41
8	Ile		
9	Glu(*O*Bzl)		
10	Lys(ClZ)	2.6	0.26
11	Val	1.6	0.15
12	Gly	2.0	0.17
13	Gln	3.7	0.29
14	Asn	5.1	0.36
15	Ile		
16	Arg(Tos)	2.0	0.13
17	Asp(*O*cHex)	5.2	0.31
18	Gly	3.9	0.22
19	Ile		
20	Ile	4.3	0.22
21	Lys(ClZ)	4.5	0.21
22	Ala	2.2	0.10
23	Gly	4.8	0.21
24	Pro		
25	Ala	3.7	0.15
26	Val		
27	Ala		
28	Val		
29	Val	3.8	0.13
30	Gly	3.5	0.12
31	Gln	3.3	0.11
32	Ala		
33	Thr(Bzl)		

[a] Total amount of the next residue in the sequence appearing at this step of the Edman degradation. Thus, at step 1, 0.27% of Trp (For)[2] was found, and at step 31 3.3% of Ala[32] was found. These numbers have all been corrected for the low-level background by subtracting the amount of the same PTH-amino acid found two or three cycles earlier.

However, the overall cumulative preview gives a reasonable estimate of the maximum number of deletions to be expected in the synthesis.

The average preview per step for the first 13 cycles of this run, which represent the last half of the chemical synthesis, was about 0.3%. Deletions (0.3–0.8%) appear to have occurred at Gln[13], Lys[7], Lys[6], Phe[5], Leu[4], and Lys[1]. In contrast, for the last half of the sequencer run there were essentially no deletions, and the coupling yields appear to have been quantitative. This means also that there was no artifactual preview at proline (Chang, 1978) generated by premature cyclization and cleavage during the derivatization with PITC. However, if a uniform coupling efficiency throughout the synthesis is assumed, then the total cumulative preview of 3.3% at step 31 leads to an average preview of 0.11% per step. On this basis we conclude that the average coupling proceeded in 99.9% yield.

Carry-over is also expected to be cummulative in this kind of analysis, and it was observed to increase gradually throughout the sequence. By step 31 the carry-over had reached 21%, and therefore the average per Edman cycle was 0.7%. The carry-over was highest for Lys(ClZ)[1], Gly[12], Arg(Tos)[16], Ile[20], and Val[26] but was not high for other residues of these same amino acids.

A random background level of 0.1–0.3% was usually observed. This effect was studied by removing the Boc group

Table IV: Amino Acid Analysis of Cecropin A(1–33)-resin Synthesis [a]

amino acid	(27–33)-resin mmol/g	(27–33)-resin mol ratio	(24–33)-resin mmol/g	(24–33)-resin mol ratio	(15–33)-resin mmol/g	(15–33)-resin mol ratio	(1–33)-resin mmol/g	(1–33)-resin mol ratio
Thr [b]	0.114	1.00	0.070	0.91	0.063	0.86	0.064	1.03
Ala	0.116	1.02	0.075	0.97	0.075	1.03	0.068	1.10
Glu	0.116	1.02	0.078	1.01	0.079	1.08	0.061	0.99
Gly	0.114	1.00	0.077	1.00	0.073	1.00	0.065	1.05
Val	0.107	0.94	0.074	0.96	0.077	1.05	0.068	1.10
Pro			0.087	1.12	0.077	1.05	0.060	0.97
Lys					0.077	1.05	0.057	0.92
Ile					0.063	0.86	0.059	0.95
Asp					0.068	0.93	0.061	0.99
Arg					0.074	1.01	0.064	1.03
Phe							0.057	0.92
Leu							0.055	0.89
Trp								
av	0.114		0.077		0.073		0.062	
av (mmol/g of styrene)	0.122		0.113		0.092		0.091	

[a] Dried samples of the peptide-resins were hydrolyzed with 12 N HCl/HOAc/phenol (2:1:1) for 24 h at 110 °C, and the amino acids were quantitated on a Beckman 121 amino acid analyzer. The observed concentrations (mmol/g) have been divided by the number of residues of each amino acid in the sample. [b] Thr(Bzl) was corrected for 20% loss on hydrolysis.

from a sample of the 1–33 peptide-resin and exhaustively acetylating the amino group. A repeat of the solid-phase sequencing experiment showed a pattern of 7 or 8 low-level peaks (0.1–0.3%) of PTH-amino acids that was nearly the same at all cycles. There was no evidence of a cumulative effect of any component and no evidence of a large chain cleavage at any point. For a further discussion of the use of the Edman degradation in the assessment of the purity of synthetic peptides see Tregear et al. (1977), Matsueda et al. (1981), and Kent et al. (1982).

Amino Acid Analyses of Peptide-Resins. The progress of the synthesis was also followed by amino acid analysis of acid-hydrolyzed samples of the peptide-resin at several stages of the chain assembly (Table IV). The mole ratios of the component amino acids were quite satisfactory in these resin hydrolysates throughout the synthesis. Threonine was approximately 20% destroyed under these conditions and was corrected accordingly. In every case Ile plus aIle was somewhat low in 24-h hydrolysates, but after 72 h it reached a ratio of approximately 1. Phe[5] and Leu[4], which were added late in the synthesis, were slightly low in these analyses, indicating some termination of chains.

It is important to note that there is no evidence of chain termination at Gln[31] or Gln[13] since the ratios of Glu in the hydrolysates were very close to 1, relative to the residues both before and after Gln. Termination by pyroglutamic acid formation would have led to an abrupt decrease in succeeding residues. The use of a rapid coupling procedure with the symmetric anhydride in DMF is thought to be responsible for this lack of termination (DiMarchi et al., 1982). The drop in total peptide chains found by step 7 is therefore not due to pyroglutamic acid formation and is attributed instead to an unusual susceptibility of the ester bond of Thr(Bzl)-Pam-resin to cleavage, especially when it is near the N terminus of the chain. Nucleophilic participation of the ether oxygen may be responsible. Chains were lost during the remaining 26 steps at a rate of about 1% per cycle, which is still much greater than that found for the loss of aliphatic residues from Pam-resin (Mitchell et al., 1976). We have subsequently observed this same effect in the synthesis of a glucagon derivative also containing a C-terminal Thr(Bzl) (G.-S. Lu and S. Mojsov, unpublished results). The rate of cleavage of Boc-Thr(Bzl) from Pam-resin by treatment with F_3CCOOH/CH_2Cl_2 (1:1) at 25 °C, however, was only 0.02% per hour, the same as for Val.

Cleavage of Synthetic Cecropin A(1–33) from Resin Support and Partial Deprotection. A sample of the fully protected synthetic peptide-resin (490 mg) was allowed to swell 30 min in 10 mL of CH_2Cl_2, filtered, and washed. The Boc group was removed by treating with 5 mL of 50% F_3CCOOH/CH_2Cl_2 containing 0.1% of ethanedithiol for 1 min and then with 5 mL of a fresh solution of the same solvent for 20 min, room temperature. After washing with CH_2Cl_2, the sample was neutralized with 5% diisopropylethylamine in CH_2Cl_2, washed with CH_2Cl_2, pumped dry, and transferred to a reaction vessel of the HF apparatus. *p*-Cresol (1.5 mL) (J. P. Tam, unpublished results) was added, and 15 mL of anhydrous HF was condensed at −78 °C. The vessel was warmed in a thermostated bath to −10 °C, and the sample was stirred at −10 °C for 30 min. It was then stirred at 0 °C for 30 min. The acid was evaporated at 0 °C with a water aspirator and then with a high-vacuum pump. The purpose of this two-step procedure was first to remove most of the protecting groups under conditions that are mild enough to avoid imide formation from Asp(*O*cHex) or acylium ion formation and subsequent acylation reactions by Glu or Asp residues but that allow the carbonium ions to decompose or be trapped by the scavenger (Feinberg & Merrifield, 1975; Baba et al., 1973). Finally, the slight elevation in temperature allows the complete removal of the more acid-resistant tosyl and cyclohexyl groups. Only the formyl group on tryptophan was stable. The product was extracted with 10% acetic acid in water, and cresol and its substitution products were removed by extraction with EtOAc. The yield based on amino acid analysis was 76%. The yields of four other similar cleavages and extractions of samples of 0.2–1.0 g were 82, 79, 70, and 77%. The HPLC analysis on a μBondapak column under conditions described under Experimental Procedures is shown in Figure 2A. The sample was applied at a high concentration, sufficient to detect impurities at the 1% level. The peak at 11.0 min is *p*-cresol, which was removed by further extraction with CH_2Cl_2. The main peak at 13.5 min accounted for 93.5% of the remaining absorbance at 225 nm. By this criterion, therefore, the average yield per synthetic cycle was 99.8%, which is in reasonable agreement with the ninhydrin and preview data. The peptide impurities were at 17.0 (5.2%) and 19.9 (1.3%) min. Ac-

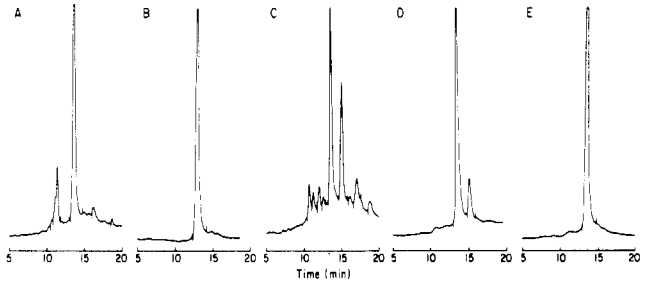

FIGURE 2: High-pressure liquid chromatographic analysis of crude and purified formylcecropin A(1–33) and cecropin A(1–33). Conditions: μBondapak column (4 × 300 mm), linear gradient of 10–50% solution B into solution A in 25 min, 2 mL/min; detection at 225 nm, 0.1 absorbance full scale; sample injections ~40 μg, overloaded in main peak. (A) Crude formylcecropin A(1–33) directly after HF cleavage and extraction into 10% HOAc; (B) purified formylcecropin A(1–33) after CM-Sepharose column; (C) crude cecropin A(1–33) after deprotection at pH 11.5; (D) crude cecropin A(1–33) after deprotection at pH 9.0; (E) purified cecropin A(1–33) after CM-Sepharose column.

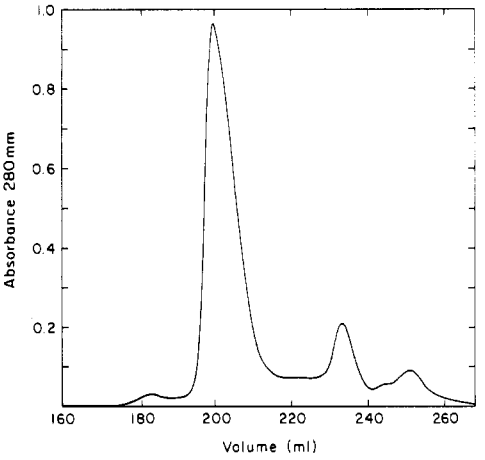

FIGURE 3: Chromatographic purification of crude formylcecropin A(1–33) directly after HF/*p*-cresol cleavage, extraction into 10% acetic acid, and lyophilization. Conditions: carboxymethyl-Sepharose column (0.9 × 46 cm), linear gradient of 0.1 to 0.5 M ammonium formate, pH 6.6, 3 mL/h; detection at 280 nm, 1.26 absorbance full scale; sample 27.8 mg.

cording to the previous data these are not deletion peptides but may be either terminated or modified peptides.

The crude formyl peptide was purified by chromatography on CM-Sepharose, Figure 3. The amount of cresol, which eluted near 1 column volume (not shown), was variable and depended on the thoroughness of the extraction procedure. The main component, peak at 198 mL, 0.35 M ammonium formate, pH 6.6, conductivity 31 mS, accounted for 86% of the total peptide absorbance at 280 nm ($\epsilon = 2.15 \times 10^3$ M^{-1} cm^{-1}). The fractions between 190 and 212 mL were combined and lyophilized, yield 21.4 mg (76%). Three additional peaks at 183, 233, and 251 mL accounted for most of the remaining material. Thus, the correspondence between the HPLC and CM-Sepharose columns on this crude, cleaved product was reasonably good. HPLC analysis (Figure 2B) of the purified formylcecropin A(1–33) from the ion-exchange column contained only trace amounts of impurities. The amino acid analysis of an HCl hydrolysate was in satisfactory agreement with theory.

Removal of N^i-Formyl Group from [Trp(For)²]Cecropin A(1–33). Initially, the formyl group was removed from tryptophan by treatment of the peptide at pH 11.5 for 3 min as recommended by Lemaire et al. (1976), followed by rapid adjustment to pH 5 with HOAc. This procedure appeared to completely remove the formyl group, but it gave rise to

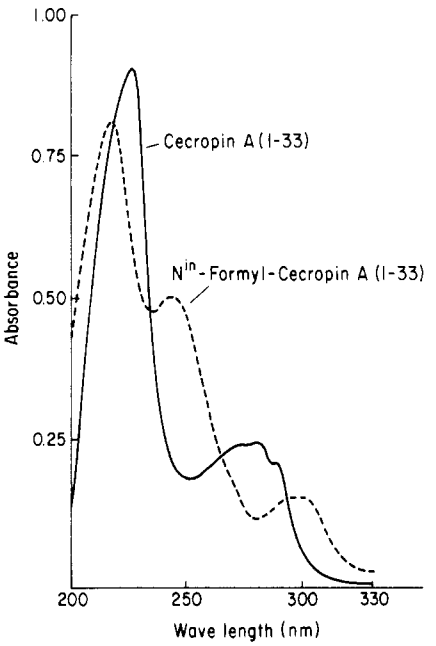

FIGURE 4: Spectra of [Trp(For)²]cecropin A(1–33) (broken line) and cecropin A(1–33) (full line), 50 μM, in 0.03 M NH_2OH, pH 9.0.

several byproducts as shown by HPLC (Figure 2C). The main component at 13.5 min corresponded closely with cecropin A(1–33) and accounted for 47% of the total absorbance at 225 mm. It was accompanied by 29% of a major byproduct at 15.0 min and smaller amounts of other byproducts. CM-Sepharose chromatography at pH 6.6 with a 0.1–0.5 M gradient of ammonium formate gave three components with peaks at tubes 56, 65, and 86, which accounted for 15, 32, and 53%, respectively, of the total recovered material by amino acid analysis. Total recovery from the column was 84%. The overall yield of cecropin A(1–33) from the fully protected peptide-resin was 22%. Since the ratios of the component amino acids in all three fractions were indistinguishable, it is probable that the fractions are composed of modified, rather than terminated peptides.

Deformylation of formylcecropin A(1–33) was then studied at room temperature in the pH range between 8 and 10 and in the presence of nucleophiles such as hydrazine and hydroxylamine (Yamashiro & Li, 1973; Ohno et al., 1972). The progress of the reaction was followed with time by scanning the spectrum from 200 to 330 nm. The peptide containing formyltryptophan absorbed with maxima at 218 ($\epsilon = 17.1 \times 10^3$), 244 ($\epsilon = 10.2 \times 10^3$), and 294 ($\epsilon = 2.9 \times 10^3$) nm (Figure 4, broken line), whereas the peptide containing deprotected tryptophan had maxima at 228 ($\epsilon = 19.6 \times 10^3$) and 282 ($\epsilon = 5.57 \times 10^3$) nm, with shoulders at 274 and 289 nm (Figure 4, full line). The decrease in absorbance at 244 and 300 nm and the increase at 282 nm were therefore a convenient way to monitor the reaction. At pH 8.0, the reaction was only about 50% complete after 3 h. At pH 9.0 the reaction was essentially complete in 2–3 h in a carbonate buffer. The addition of 0.03 M NH_2NH_2 to scavenge formyl groups accelerated the reaction, and no further absorbance changes were observed after 60 min. Finally, hydroxylamine hydrochloride was adjusted to pH 9.0 with NH_4OH and added in a final concentration of 0.03 M to the formyl-peptide sample. Under these conditions the reaction was complete in approximately 2 h (Table V).

In order to observe the appearance of new components during the deformylation reaction, we ran aliquots in the HPLC system. Formylcecropin A(1–33) and cecropin A(1–

Table V: Spectral Changes during Deformylation of Formylcecropin A(1–33)[a]

time (min)	absorbance 244 nm	282 nm	300 nm
0	0.476	0.100	0.135
8.5	0.402	0.111	0.112
30	0.293	0.160	0.082
60	0.186	0.186	0.073
140	0.115	0.247	0.050
180	0.115	0.253	0.055

[a] Formylcecropin A(1–33), 0.166 mg/mL in 0.030 M hydroxylamine, pH 9.0.

Table VI: Summary of Workup of Cecropin A(1–33)

step	mmol/g of peptide-resin[a]	yield per step (%)	cumulative yield (%)
(1) Boc-peptide-resin	0.062	100	100
(2) HF cleavage, HOAc extraction	0.047	76	76
(3) CM-Sepharose, main peak, isolated	0.036	76	58
(4) removal of formyl, pH 9	0.030	84	49
(5) CM-Sepharose, main peak, isolated	0.024	80	39

[a] Determined by amino acid analysis.

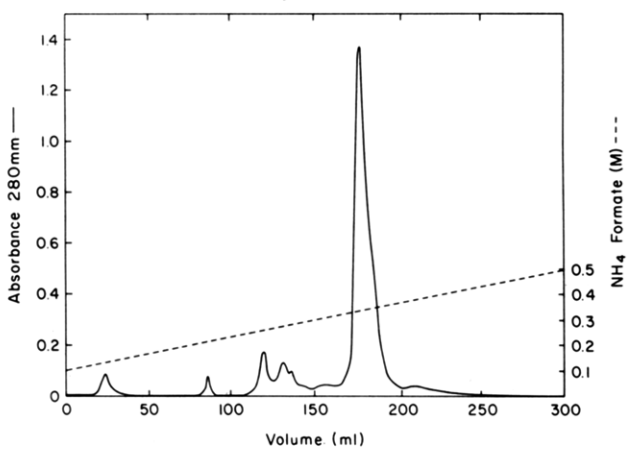

FIGURE 5: Chromatographic purification of cecropin A(1–33) after removal of the N^{i}-formyl group at pH 9.0. Conditions similar to those in Figure 3.

33) were not resolved in this system, but loss of the Trp(For) residue was readily confirmed by monitoring the chromatogram at 300 nm. The appearance of byproducts was best monitored at 225 nm. A single new peak at 15.06 min began to appear after a few min in 0.03 M NH_2OH, pH 9.0, and increased to 15.6% over a period of 2–3 h (Figure 2D; compare with Figure 2C). Some transfer of the formyl group to α- or ϵ-amino groups is expected under these conditions (Yamashiro & Li, 1973). The corresponding experiment with NH_2NH_2 gave more byproduct. It was subsequently found that removal of the formyl group by 1.0 M NH_2OH, pH 9.0, was complete in less than 2 h, and most importantly, no HPLC-detectable byproduct (<0.5%) was produced after 3 h of treatment.

A 50-mg sample of purified formylcecropin A(1–33) was deprotected by the pH 9.0, 0.03 M hydroxylamine procedure, and the byproduct was removed by chromatography on a 0.9 × 46 cm CM-Sepharose column in the 0.1–0.5 M ammonium formate, pH 6.6, system (Figure 5). The fractions between

FIGURE 6: $NaDodSO_4$ gel electrophoresis of synthetic cecropin A-(1–33): (lane A) synthetic cecropin A(1–33), 20 μg; (lane B) in descending order, soybean trypsin inhibitor (20095 daltons), lysozyme (14314 daltons), glucagon (3483 daltons) plus insulin B-chain (3400 daltons), and insulin A-chain (2320 daltons). The cathode is at the top.

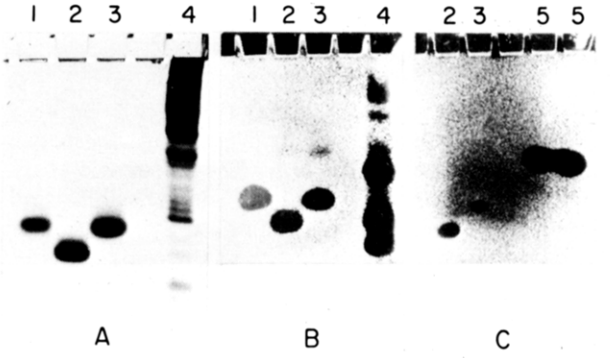

FIGURE 7: Three gels from acid electrophoresis of formylcecropin A(1–33) (lanes 1), natural cecropin A (lanes 2), synthetic cecropin A(1–33) (lanes 3), immune hemolymph from *H. cecropia* pupae as reference (lanes 4), and methyl green marker dye (lanes 5). Gels A and B were run simultaneously; gel C was run separately in the same apparatus. The size differences are due to the differences in development. All samples were run in 15% acrylamide–0.2% bis-(acrylamide) gels, pH 4, according to Hultmark et al. (1980), with the cathode at the bottom. Gel A was stained for proteins, gel B was overlaid with viable *E. coli*, and gel C was overlaid with viable *B. megaterium*. The amounts applied were (gel A) 20 μg of the respective cecropins and 15 μL of hemolymph, (gel B) 0.4 μg of cecropin A, 2 μg of cecropin A(1–33), 4 μg of formylcecropin A(1–33), and 4 μL of hemolymph, (gel C) 4 μg of cecropin A and 20 μg of cecropin A(1–33).

165 and 200 mL were combined, dialyzed, and lyophilized, yield 33 mg (67%). The workup of one run of the synthetic peptide is shown in Table VI.

The purified product showed by HPLC a major component at 13.5 min and only traces of impurity (Figure 2E). The purified product gave a single Coomassie blue band after $NaDodSO_4$ gel electrophoresis, which migrated with approximately the expected (3565) molecular weight when compared with those of glucagon and insulin A and B chain markers (Figure 6). Homogeneity was further examined by acid–polyacrylamide gel electrophoresis. Synthetic cecropin A(1–33), synthetic formylcecropin A(1–33), and natural cecropin A each showed a single strong Coomassie blue band, followed by a faint trailing (Figure 7, gel A).

In a parallel separation, the antibacterial activity toward *E. coli* (gel B) and *B. megaterium* (gel C) was found to have

Table VII: Amino Acid Analysis of Purified Synthetic Cecropin A(1–33)

amino acid	residue no.	mol ratio HCl 24 h	HCl 48 h	HCl 72 h	$MeSO_3H$, 24 h
Asp	2	1.02	1.02	1.02	1.00
Thr	1	0.99	0.95	0.92[a]	1.10
Glu	3	1.04	1.00	1.02	1.06
Pro	1	0.71	0.84	0.91	1.06
Gly	4	1.00	1.01	1.01	0.97
Ala	4	1.00	1.03	1.03	0.92
Val	4	0.86	0.98	1.00	0.83[b]
Ile[c]	4	0.80	0.92	0.96	0.67[b]
Leu	1	0.90	0.94	0.97	0.89
Phe	1	1.11	1.05	1.09	1.04
Lys	6	0.94			0.99
Arg	1	0.83	1.02	1.00	0.99
Trp	1				1.00
total	33			av 1.00	av 1.00

[a] 24-h value used for average. [b] Omitted from average. [c] Includes alloisoleucine.

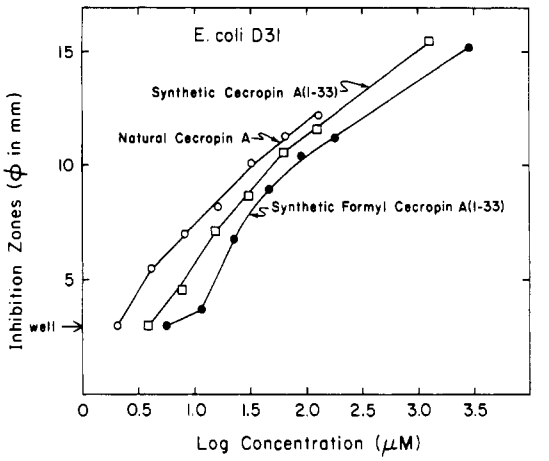

FIGURE 8: Antibacterial activity of synthetic cecropin A(1–33) (open squares), synthetic [Trp(For[2])cecropin A(1–33) (filled circles), and natural cecropin A (open circles). Samples of 4×10^{-6} to 1.4×10^{-3} M concentration were placed in small wells punched in thin agar plates seeded with *E. coli* D31. The inhibition zones around the wells were measured after an overnight incubation at 37 °C.

the same mobility as the stained bands. In the *E. coli* test plate, the weak antibacterial activity with a lower mobility (lanes 2 and 3, gel B) is thought to be due to the known antibacterial impurity present in the added methyl green tracker dye (compare with lane 5, gel C).

Aliquots of the purified peptide were hydrolyzed in 6 N HCl by heating in evacuated sealed tubes at 110 °C for 24, 48, and 72 h, and another aliquot was hydrolyzed in 4 N methanesulfonic acid at 110 °C for 24 h. The amino acid analyses were in good agreement with theory and are summarized in Table VII. Tryptophan was quantitatively recovered after $MeSO_4$ hydrolysis. Note that cecropin A contains Val–Val and Ile–Ile sequences and required 72-h hydrolysis in 6 N HCl at 110 °C for complete release of these two amino acids.

Antibacterial Activity. Natural cecropin A, synthetic cecropin A(1–33), and [Trp(For)[2]]cecropin A(1–33) were assayed for activity against *E. coli* D31 by mic values and by measuring the diameters of the zones of inhibition on thin agar plates. The first of the methods gave in two determinations with different 2-fold dilutions the following mic values: for natural cecropin A, 0.3–0.6 and 0.5–1.0 μM; for synthetic cecropin A(1–33), 0.6–1.3 and 0.5–1.0 μM; for formylated cecropin A(1–33) 1.3–2.6 and 1.0–2.0 μM. Thus there was about a 2-fold reduction in antibacterial activity between the natural, the synthetic A(1–33), and the formylated A(1–33).

The inhibition zone assay (Figure 8) showed a similar relationship between the three compounds. For both assays the recorded differences are close to the limits of sensitivity of the method. The dose–response curves in Figure 8 were approximately parallel over most of the assay range. However, at very low concentrations the peptides deviated from the linear logarithmic relationship, indicating a threshold effect, and the observed deviation was greater for the synthetic peptides than for the larger natural peptide.

Table VIII gives interpolated values from similar experiments with two peptides against three other bacteria. With the two insect pathogens *Xenorhabdus nematophilus* and *Pseudomonas aeruginosa* the shortened peptide gave inhibition zones of approximately half the diameter of those produced by an equal weight of cecropin A. In this assay the zone of inhibition of *B. megaterium* did not extend beyond the diameter of the sample well, indicating an apparent lack of activity even at 0.31 μg/μL. However, on the basis of electrophoresis experiments (Figure 7, gel C) there was detectable activity against *B. megaterium*. A 20-μg aliquot of synthetic cecropin A(1–33) gave a small but clearly discernable zone of inhibition, which migrated less toward to cathode than the native peptide (4 μg) as expected for a peptide containing one less lysine and one more carboxyl at the C terminus.

Discussion

An examination of the sequence of cecropin A suggested that it might be a rather stern test for any synthetic method. It has very hydrophobic segments containing, for example, pairs of β-branched Val and Ile, which for steric reasons might be expected to lead to slow and perhaps incomplete coupling reactions. The high potential for α-helix formation might also give rise to structures that are rigid and relatively inaccessible for reaction in organic solvents. A fragment synthesis in solution might therefore result in a number of insoluble intermediates that would be difficult to handle.

Recent studies on the solvation and swelling properties of resin-bound peptides (Sarin et al., 1980; Kent & Merrifield, 1981) have convinced us that difficulties with solubility would be greatly reduced in a solid-phase synthesis, but nevertheless, the cecropins appeared to be potentially difficult molecules

Table VIII: Effect of Synthetic Cecropin A(1–33) and Natural Cecropin A on Growth of Several Bacteria Strains[a]

cecropin sample	concn (μg/μL)	inhibition zone (mm) *E. coli* K12 and D31	*X. nematophilus* Xn 21	*P. aeruginosa* OT 97	*B. megaterium* Bm 11
natural 1–37	0.031	7.1	4.3	2.7	5.0
	0.31	11.9	12.0	6.6	8.4
synthetic 1–33	0.031	4.9	2.7	2.7	2.7
	0.31	7.1	7.1	3.5	2.7

[a] Bacteria concentration 1.3×10^4 cells/mL. 3 μL of sample was added to 2.7-mm wells.

to synthesize. For that reason we planned to monitor the course of the synthesis at every step by using the newly developed quantitative ninhydrin reaction. To maximize solvation and to promote rapid and complete coupling reactions, we routinely carried out a double coupling at each step using both symmetric anhydrides and hydroxybenzotriazole esters and alternating between methylene chloride and dimethylformamide as the solvent.

The data showed that this synthesis of cecropin A(1–33) actually went in very high yield at every step and no serious difficulty was encountered in assembling the peptide chain. On the basis of ninhydrin monitoring, amino acid analysis, and preview sequencing the coupling yields averaged approximately 99.8% per step. The most serious problem was the loss of peptide chains from the resin, due to decreased stability of the Thr(Bzl)-resin bond. The loss of chains does not affect the purity of the product, but it reduced the overall yield of protected peptide-resin to about half after the 33 synthetic steps. Sequencing data showed that 97% of the remaining peptide chains on the resin had the correct sequence. The cleavage yields were high, and 93.5% of the crude cleaved product was shown to be the desired peptide, indicating that only 3–4% of byproducts were introduced during the HF reaction. The peptide, still containing a formyl protecting group on the tryptophan, was readily purified by ion-exchange chromatography on CM-Sepharose. The formyl group was quantitatively removed from the tryptophan at pH 9 in the presence of hydroxylamine to avoid the transfer of part of the formyl groups to either the α-amino group on Lys[1] or to the ϵ-amino group on one of the six lysines (Yamashiro & Li, 1973). At high concentration (1 M) of the trapping agent no detectable amount of byproduct was formed. The fully deprotected cecropin A(1–33) was isolated in purified form on CM-Sepharose. Throughout the workup of this peptide there were relatively large losses during each chromatographic and lyophilization step, giving an overall yield for the cleavage, deprotection, two chromatographic purification steps, and isolation of 39%. Adsorption and aggregation tendencies of the molecule may be responsible for these losses. The total yield of cecropin A(1–33) from the initial 2.2 g of aminomethyl-resin was calculated to be 279 mg. The final product was homogeneous by ion-exchange chromatography, HPLC, and gel electrophoresis, and it gave good amino acid analyses. We believe it is of high purity and that it has the correct structure.

It is probable that the mechanism of the bacteriolytic action of the cecropins is due in part to surface active properties of the peptides. An examination of the sequences of cecropins A and B shows that they contain long hydrophobic regions and that there is a very basic segment at the N terminus. As pointed out by Steiner et al. (1981), this is in contrast with the lytic and toxic peptide melittin, in which the relative positions of the hydrophobic and basic segments are reversed. In addition, in a recent study on the secondary structure of cecropins A and B, Steiner (1982)[2] has concluded from CD measurements that these peptides exist largely as random coils in dilute aqueous buffer but fold into more helical conformations in hydrophobic environments. He has predicted from sequence data, according to Chou–Fasman (1974, 1977) analysis, and model building that residues 1–11 probably form an amphipathic helix (Segrest et al., 1974).

We find that circular dichroism measurements show very little structure for synthetic cecropin A(1–33) in water, but

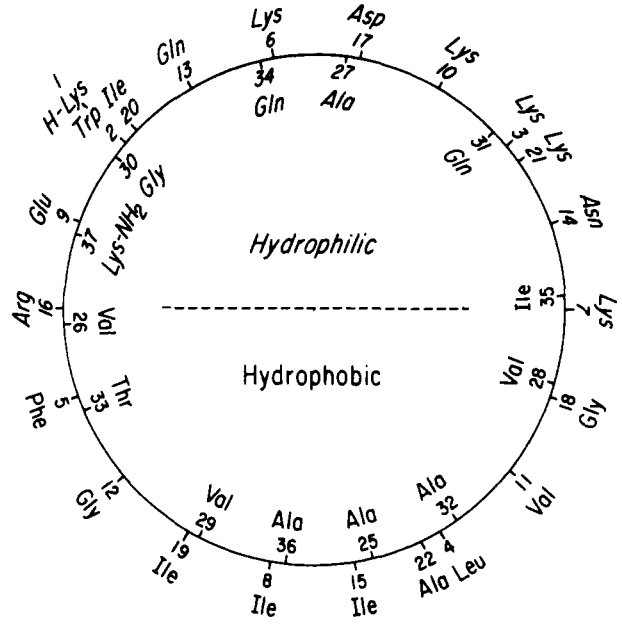

FIGURE 9: Amphipathic α helix for cecropin A. The axial projection of the peptide in two α-helical segments shows the distribution of the charged and hydrophilic residues on the top surface and the hydrophobic residues on the bottom surface. The C-terminal lysine is shown as an amide.

in trifluoroethanol the molecule assumes a conformation with about 50% helix ($\theta_{222} = -1.5 \times 10^4$ deg cm^2 dmol^{-1}). Chou–Fasman calculations show $\langle P_\alpha \rangle$ values greater than 1 for the segments 2–11, 16–22, and 25–33, although in water the 16–22 segment is predicted to be a random coil and 25–33 is expected to be balanced between a potential β sheet and an α helix. a β bend is indicated for residues 12–15, and the strong helix disrupters are present at Gly[23] and Pro[24]. It seems quite reasonable, however, that the molecule, when in contact with a hydrophobic membrane, might be induced to exist as an extended helix for most of its length, with only a break around Gly[23] and Pro[24].

When plotted as an Edmundson α-helical wheel (Schiffer & Edmundson, 1967), cecropin A(1–33) and cecropin A provide remarkable examples of the amphipathic α helix (Segrest et al., 1974; Assmann & Brewer, 1974). The structure represented in Figure 9 is a planar projection from the N terminus down the axis of the entire cecropin A molecule when constructed as two ideal α-helical segments. The residues of the N-terminal helix (2–22) are drawn on the outside of the circle, and those of the C-terminal helix (25–37) are shown on the inside. The first amphipathic helix is depicted as beginning with Trp[2], since Lys is usually not found at the N terminus of natural protein helices, and proceeds in a regular way to Ala[22]. After disruption by Gly[23] and Pro[24] a new helical segment begins and for convenience is drawn coaxial with the first and adjusted in an arbitrary way to maintain the hydrophobic and hydrophilic surfaces of the two helices in the same orientation. In this conformation there are on the top side of the cylindrical surface 14 hydrophilic residues (9 charged and 5 neutral) and only 3 hydrophobic residues. On the bottom surface there are 15 hydrophobic residues, 2 glycines, and no other hydrophilic or charged residues.

Whether or not such a model bears any relation to the actual mechanism of antibacterial action of the cecropins remains to be determined. However, the outstanding successes with studies on apolipoproteins (Morrisett et al., 1977; Sparrow, 1975; Fukushima et al., 1979) and with melittin (DeGrado et al., 1981) suggest that the model will also play an important

[2] We thank Dr. Steiner for disclosing to us the contents of his manuscript before publication.

role in deducing the mode of action of the cecropins.

Natural cecropin A contains a blocked C-terminal tetrapeptide (-Gln-Ile-Ala-Lys-X, residues 34–37), which is absent in the synthetic cecropin A(1–33). The difference in electrophoretic mobility between the two substances (Figure 7) was in fact how the presence of the terminal sequence in the natural molecule was deduced. When the natural and synthetic compounds were compared on a molar basis for activity against *E. coli* D31, there was a small but reproducible difference in potency with both of the methods used. In the inhibition zone assay (Figure 8) there was a nearly linear relation between the diameter of the zones and the logarithm of the initial concentration of the compounds over a wide range. However, at the lower concentrations a deviation from this linear dependence, due to a threshold effect, was always observed and was more pronounced for the shorter 1–33 analogue. The presence of a formyl group on the indole nitrogen of tryptophan at position 2 increased the differences in potency and threshold already seen between the natural and unprotected synthetic compound.

The findings described here indicate that the last four amino acid residues and the carboxyl blocking group at the C terminus of cecropin A do not play a unique role in the antibacterial action, as was to be expected since cecropin B is equally active against *E. coli* even though it has a different sequence in this region. A nonspecific effect of residues in this tetrapeptide region does play a role in the killing of *E. coli* and appears to be very important for effective killing of *B. megaterium* (Table VIII, Figure 7). These effects may be strongly dependent on the tendency of the peptide to conform to an amphipathic α-helical structure, and this property may be very sensitive to small changes in the composition of the peptide; a thesis that can be examined experimentally.

It was possible to demonstrate by an antibacterial assay conducted after electrophoretic fractionation (Figure 7) that A(1–33) has a bactericidal effect on the Gram-positive organism *B. megaterium*. However, a 5-fold increase in concentration (compared to natural cecropin A) was necessary. Although natural cecropin A has been isolated in a highly pure form, the possibility of contamination by lysozyme known to be present in the hemolymph has made the interpretation of its apparent antibacterial spectrum uncertain. Because the assay of A(1–33) was with a synthetic peptide, it cannot involve any contamination with lysozyme, and it is, therefore, the first convincing demonstration that cecropins can act on a lysozyme-sensitive Gram-positive bacterium.

Acknowledgments

We thank Dr. Håkan Steiner and David Cowburn for several discussions and Marie LeDoux for the amino acid analyses.

References

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

Baba, T., Sugiyama, H., & Seto, S. (1973) *Chem. Pharm. Bull. 21*, 207–209.

Bhown, A. S., Mole, J. E., Weissinger, A., & Bennett, J. C. (1978) *J. Chromatogr. 148*, 532–535.

Boman, H. G., & Hultmark, D. (1981) *Trends Biochem. Sci. 6*, 306–309.

Boman, H. G., & Steiner, H. (1981) *Curr. Top. Microbiol. Immunol. 94–95*, 75–91.

Chang, J. Y. (1978) *FEBS Lett. 91*, 63–68.

Chou, P. Y., & Fasman, G. D. (1974) *Biochemistry 13*, 222–245.

Chou, P. Y., & Fasman, G. D. (1977) *J. Mol. Biol. 115*, 135–175.

De Grado, W. F., Kêzdy, F. J., & Kaiser, E. T. (1981) *J. Am. Chem. Soc. 103*, 679–681.

DiMarchi, R. D., Tam, J. P., Kent, S. B. H., & Merrifield, R. B. (1982) *Int. J. Pept. Protein Res. 19*, 88–93.

Erickson, B. W., & Merrifield, R. B. (1972) *J. Am. Chem. Soc. 95*, 3757–3763.

Feinberg, R. S., & Merrifield, R. B. (1975) *J. Am. Chem. Soc. 97*, 3485–3496.

Fukushima, D., Kupferberg, J. P., Yokoyama, S., Kroon, D. J., Kaiser, E. T., & Kêzdy, F. J. (1979) *J. Am. Chem. Soc. 101*, 3703–3704.

Gisin, B. F., & Merrifield, R. B. (1972) *J. Am. Chem. Soc. 94*, 3102–3106.

Gutte, B., & Merrifield, R. B. (1971) *J. Biol. Chem. 246*, 1922–1941.

Hagenmaier, H., & Frank, H. (1972) *Hoppe-Seyler's Z. Physiol. Chem. 353*, 1973–1976.

Hultmark, D., Steiner, H., Rasmuson, T., & Boman, H. G. (1980) *Eur. J. Biochem. 106*, 7–16.

Kent, S. B. H., & Merrifield, R. B. (1981) in *Peptides 1980*, (Brunfeldt, K., Ed.) pp 328–333, Scriptor, Copenhagen, Denmark.

Kent, S. B. H., Mitchell, A. R., Engelhard, M., & Merrifield, R. B. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 2180–2184.

Kent, S. B. H., Riemen, M., LeDoux, M., & Merrifield, R. B. (1982) in *Methods in Protein Sequence Analysis* (Elzinga, M., Ed.) pp 205–213, Humana Press, Clifton, NJ.

König, W., & Geiger, R. (1970) *Chem. Ber. 103*, 788–798.

Laursen, R. A. (1971) *Eur. J. Biochem. 20*, 89–102.

Lemaire, S., Yamashiro, D., & Li, C. H. (1976) *J. Med. Chem. 19*, 373–376.

Matsueda, G. R., Haber, E., & Margolies, M. N. (1981) *Biochemistry 20*, 2571–2580.

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

Merrifield, R. B., Stewart, J. M., & Jernberg, N. (1966) *Anal. Chem. 38*, 1905–1914.

Mitchell, A. R., Erickson, B. W., Ryabtsev, M. N., Hodges, R. S., & Merrifield, R. B. (1976) *J. Am. Chem. Soc. 98*, 7357–7362.

Mitchell, A. R., Kent, S. B. H., Engelhard, M., & Merrifield, R. B. (1978) *J. Org. Chem. 43*, 2845–2852.

Mojsov, S., Mitchell, A. R., & Merrifield, R. B. (1980) *J. Org. Chem. 45*, 555–560.

Morrisett, J. D., Jackson, R. L., & Gotto, A. M. (1977) *Biochim. Biophys. Acta 472*, 93–133.

Niall, H. D., Tregear, G. W., & Jacobs, J. (1972) in *Chemistry and Biology of Peptides* (Meienhofer, J., Ed.) pp 695–699, Ann Arbor Press, Ann Arbor, MI.

Ohno, M., Tsukamoto, S., Sato, s., & Izumiya, N. (1972) *Bull. Chem. Soc. Jpn. 46*, 3280–3285.

Previero, A., Coletti-Previero, M. A., & Cavadore, J. C. (1967) *Biochim. Biophys. Acta 147*, 453–461.

Sarin, V. K., Kent, S. B. H., & Merrifield, R. B. (1980) *J. Am. Chem. Soc. 102*, 5463–5470.

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

Schiffer, M., & Edmundson, A. B. (1967) *Biophys. J. 7*, 121–135.

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

Simpson, R. J., Neuberger, M. R., & Liu, T. Y. (1976) *J. Biol. Chem. 251*, 1936–1940.

Spangenberg, R., Thamm, P., & Wünsch, E. (1971) *Hoppe-Seyler's Z. Physiol. Chem. 352*, 655–656.
Sparrow, J. T., Morrisett, J. D., Pownall, H. J., Jackson, R. L., & Gotto, A. M., Jr. (1975) in *Peptides* (Walter, R., & Meienhofer, J., Eds.) pp 597–602, Ann Arbor Science, Ann Arbor, MI.
Steiner, H. (1982) *FEBS Lett. 137*, 283–287.
Steiner, H., Hultmark, D., Engström, A., Bennich, H., & Boman, H. G. (1981) *Nature (London) 292*, 246–248.
Tam, J. P., Kent, S. B. H., Wong, T. W., & Merrifield, R. B. (1979a) *Synthesis*, 955–957.
Tam, J. P., Wong, T. W., Riemen, M. W., Tjoeng, F. S., & Merrifield, R. B. (1979b) *Tetrahedron Lett.*, 4033–4036.
Tregear, G. W., van Rietschoten, J., Sauer, R., Niall, H. D., Keutmann, H. T., & Potts, J. T., Jr. (1977) *Biochemistry 16*, 2817–2823.
Yamashiro, D., & Li, C. H. (1973) *J. Org. Chem. 38*, 2594–2597.

A Proton Nuclear Magnetic Resonance Investigation of Histidyl Residues in Human Normal Adult Hemoglobin†

Irina M. Russu, Nancy Tseng Ho, and Chien Ho*

ABSTRACT: High-resolution proton nuclear magnetic resonance (NMR) spectroscopy at 250 MHz has been used to titrate 22 individual surface histidyl residues (11 per $\alpha\beta$ dimer) of human normal adult hemoglobin in both the deoxy and the carbon monoxy forms. The proton resonances of β2, β143, and β146 histidyl residues are assigned by a parallel ^{1}H NMR titration of appropriate mutant and chemically modified hemoglobins. The pK values of the 22 histidyl residues investigated are found to range from 6.35 to 8.07 in the deoxy form and from 6.20 to 7.87 in the carbon monoxy form, in the presence of 0.1 M Bis-Tris or 0.1 M Tris buffer in D_2O with chloride ion concentrations varying from 5 to 60 mM at 27 °C. Four histidyl residues in the deoxy form and one histidyl residue in the carbon monoxy form are found to have proton nuclear magnetic resonance titration curves that deviate greatly from that predicted by the simple proton dissociation equilibrium of a single ionizable group. The proton nuclear magnetic resonance data are used to ascertain the role of several surface histidyl residues in the Bohr effect of hemoglobin under the above-mentioned experimental conditions. Under these experimental conditions, we have found that (i) the β146 histidyl residues do not change their electrostatic environments significantly upon binding of ligand to deoxyhemoglobin and, thus, their contribution to the Bohr effect is negligible, (ii) the β2 histidyl residues have a negative contribution to the Bohr effect, and (iii) the total contribution of the 22 histidyl residues investigated here to the Bohr effect is, in magnitude, comparable to the Bohr effect observed experimentally. These results suggest that the molecular mechanism of the Bohr effect proposed by Perutz [Perutz, M. F. (1970) *Nature (London) 228*, 726–739] is not unique and that the detailed mechanism depends on experimental conditions, such as the solvent composition.

High-resolution proton nuclear magnetic resonance (NMR)[1] spectroscopy is the only experimental technique currently available capable of monitoring the environments and the conformations of individual amino acid residues of a protein molecule in solution. Extensive research carried out in the last decade has shown that this technique is most suitable for the study of the structure–function relationship of hemoglobin (Hb) in solution [for a recent review, see, for example, Ho & Russu (1981)]. A large variety of spectroscopic probes for the Hb molecule are available when using ^{1}H NMR. They extend over the entire Hb molecule from protons situated close to the iron atoms and in the heme pockets (Ho et al., 1973, 1978; Lindstrom & Ho, 1973; Shulman et al., 1975; Takahashi et al., 1980) to those at the $\alpha_1\beta_1$ and $\alpha_1\beta_2$ subunit interfaces (Fung & Ho, 1975; Viggiano & Ho, 1979) and further to those situated on the surface of the molecule (Kilmartin et al., 1973; Fung et al., 1975; Ho et al., 1976; Russu et al., 1980). Most of these spectroscopic probes belong to those amino acid residues that have been proposed, on the basis of X-ray diffraction results, to play key roles in the function of Hb (Baldwin, 1975). Among them, the interest of the present work is concentrated on the surface histidyl (His) residues of Hb.

Several His residues of human normal adult hemoglobin (Hb A) have been proposed, on the basis of X-ray diffraction data and the results on mutant and chemically modified hemoglobins, to play an important role in the variation of the oxygen affinity with pH, known as the Bohr effect (Perutz, 1970; Perutz et al., 1980). The involvement of these surface His residues in the Bohr effect originates from the changes in their electrostatic environments accompanying the change in the conformation of the Hb molecule upon ligation. For example, Perutz (1970) has proposed that the carboxyl-terminal β146 His is one of the residues responsible for the

† From the Department of Biological Sciences, Carnegie-Mellon University, Pittsburgh, Pennsylvania 15213. *Received February 17, 1982.* This work was supported by research grants from the National Institutes of Health (HL-24525) and the National Science Foundation (PCM 78-25818). The MPC-HF 250-MHz NMR spectrometer in the Pittsburgh NMR Facility is supported by a research grant from the National Institutes of Health (RR-00292). This paper was presented in part at the Symposium on Interaction between Iron and Proteins in Oxygen and Electron Transport, Airlie, VA, April 13–18, 1980.

[1] Abbreviations: NMR, nuclear magnetic resonance; Hb, hemoglobin; HbCO, (carbon monoxy)hemoglobin; Hb A, human normal adult hemoglobin; des-His-Hb, des-β146-histidine-hemoglobin; HbO_2, oxyhemoglobin; ppm, parts per million; Bis-Tris, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane; Tris, tris(hydroxymethyl)aminomethane; 2,3-DPG, 2,3-diphosphoglycerate; IHP, inositol hexaphosphate; Ac-His-MA, *N*-acetyl-L-histidine methylamide; DEAE, diethylaminoethyl.

0006-2960/82/0421-5031$01.25/0