Cupiennin 1, a New Family of Highly Basic Antimicrobial Peptides in the Venom of the Spider Cupiennius salei (Ctenidae)*

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Received for publication, November 20, 2001
Published, JBC Papers in Press, January 15, 2002, DOI 10.1074/jbc.M111099200

A new family of antimicrobial peptides was isolated from the venom of Cupiennius salei. The peptides were purified to homogeneity, and the sequence of cupiennin 1a was determined by Edman degradation: GFGALFR-FLAKVAKTVAKQAAEQGAKYVVKME-NH2. The amino acid sequences of cupiennin 1b, c, and d were obtained by a combination of sequence analysis and mass spectrometric measurements of comparative tryptic peptide mapping. All peptides consist of 35 amino acid residues and are characterized by a more hydrophobic N-terminal chain region and a C terminus composed preferentially of polar and charged residues. The total charge of all cupiennins calculated under physiological conditions is +8, and their C terminus, formed by a glutamic acid residue, is amidated. Conformational studies of the peptides revealed a high helix forming potential. Antimicrobial assays on bacteria with cupiennin 1a, 1d, and synthesized cupiennins 1a* and 1d* showed minimal inhibitory concentrations for bacteria in the submicromolar range. Their lytic effect on human red blood cells was lower by a factor of 8 to 14 than the highly hemolytic melittin. Cupiennin 1a, 1b, 1d, 1a*, and 1d* showed pronounced insecticidal activity. The immediate biological effects and the structural properties of the isolated cupiennins indicate a membrane-destroying mode of action on prokaryotic as well as eukaryotic cells.

Antimicrobial peptides are ubiquitous in nature as a part of the innate immune system and host defense mechanism. They are produced by various species, both in prokaryotic and eukaryotic cells (for reviews, see Refs. 1–5). Many of these peptides act within minutes by a cell-lytic/ionicophoric, nonstereo-selective mechanism against a broad spectrum of bacteria (6), protozoa (7, 8), filamentous fungi (9, 10), tumor cells (11), and enveloped viruses (12). Additional mechanisms to kill bacteria by acting stereospecifically on molecular targets have been described (13–15).

As a selective response to microbial invasion, several antimicrobial peptides have been identified in the hemolymph (e.g. hemocytes) of insects (16), spiders (17), and scorpions (18). Only a few antimicrobial peptides are exclusively and constitutively present in the venom glands of insects, namely melittin (19) and crabolin (20), and in scorpions, hadurin (21) and scorpion (22). In 1989 the first report on bactericidal peptides in spider venom (Lycosa singoriensis) was published (23), later, lycotoxins were isolated from Lycosa carolinensis (24).

Spiders are hunting predators and use paralytic venoms to immobilize their prey. Most components in their venoms act on the nervous system and are enzymatically active causing cell membrane disruption and tissue necrosis. The venom of Cupiennius salei, a hunting spider found in Central America, yielded both neurotoxically active peptides (named CSTX-1 to CSTX-13) (25) and bactericidal peptides (26). To differentiate between the two groups of peptides, we named the family of antimicrobial compounds cupiennins.

Here, the purification of the cupiennin family 1 composed of four very similar peptides, named cupiennin 1a, 1b, 1c, and 1d, is reported. Cupiennin 1a is identical with peptide 8, and cupiennin 1d is identical with peptide 7 in Ref. 26. Sequence analysis of the highly cationic peptides and helix projection revealed a unique structure distinctly different from that of other potentially helical cationic peptides isolated so far. The highly antimicrobial, hemolytic, and insecticidal activity and the structural properties indicate a membrane-disturbing function of the cupiennins on prokaryotic as well eukaryotic cells. These peptides may provide a powerful tool in analyzing the structural prerequisites of antimicrobial selectivity and general cytolytic membrane action. In the light of the massive increase of multi-drug-resistant bacterial infections, further structural investigations of cupiennins may prove helpful in the development of new antimicrobial peptide pharmaceuticals.

EXPERIMENTAL PROCEDURES

Chemicals—Trifluoroacetic acid (for protein sequencing), TFE,2 and acetonitrile (LiChrosolv® for chromatography) were purchased from Merck. Melittin (amidated) was purchased from Tocris (Anawa Trading SA, Zürich, Switzerland) and magainin 2 (not amidated) from Sigma.

Isolation of Toxins—C. salei (Ctenidae) spider maintenance, venom collection by an electrical milking procedure, separation of venom by gel filtration, cationic exchange chromatography, and reversed-phase HPLC were performed as described previously (25). Briefly, 450 μl of

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crude venom was fractionated into nine 50-µl aliquots and diluted with 150 µl of 200 mM ammonium acetate buffer, pH 5.5 (buffer A). The diluted venom was separated on a Superdex 75 HR 10/30 column (Amersham Biosciences, Inc.) in buffer A, and fractions were collected as noted on the chromatogram (see Fig. 1A). Further separation of the pooled fractions was achieved by buffer or mixed with TFE to give a final concentration of 50 µM and the desired solvent composition. Measurements were carried out on a J-720 spectrometer in 0.1-cm cells between 195 and 260 nm at room temperature (Jasco). Each spectrum was the average of five scans. The baseline was subtracted. The helicity (α) of cupiennin 1a, 1a*, and 1d* was determined from the mean residue ellipticity at 222 nm according to the equation: α (%) = ([θ]222 + 2340) / 1000/ −3000 (29).

Antimicrobial Cupiennins—Bacteria (Escherichia coli ATCC 25922; Staphylococcus aureus ATCC 29213; Enterococcus faecalis ATCC 29212, Pseudomonas aeruginosa ATCC 27853) were cultured in Mueller-Hinton broth. Determination of the minimal inhibitory concentration for the cationic antimicrobial peptides was performed using a 2-fold microtiter broth dilution assay (30). Mueller-Hinton broth was used to dilute the bacterial inoculum, which was prepared from mid-log phase cultures to give a final concentration of 1.7–3.8 × 10^8 colony-forming units/ml in the wells. First, 100 µl of the bacteria dilution was added to the wells followed by 10 µl of the test peptides in 0.01% acetic acid, 0.2% bovine serum albumin. The peptides (0.04–100 µM), a nontreated growth control, and a sterility control were tested in triplicate. The microtiter plates were incubated at 37°C for 24 h. The content of the first four wells showing no visible growth of bacteria (measured as an increase of optical density at 630 nm) were plated out on blood agar plates and incubated at 37°C for 18 h. Minimal inhibitory concentrations (MIC) are expressed as intervals of concentrations, [a] – [b], where [a] is the highest concentration of peptide at which bacteria still grow and [b] the lowest concentration causing 100% growth inhibition (no colony forming bacteria estimated after additional plating of 91% of the tested bacteria suspension).

Hemolytic Assay—The hemolytic activity of cupiennin 1a, 1a*, 1b, 1d, 1d*, magainin 2, and mellitin was determined using fresh human erythrocytes. 1 ml of citrated blood was washed four times with 6 ml of PBS buffer (50 mM sodium phosphate buffer, 150 mM NaCl, pH 7.2) and centrifuged (900 × g) for 6 min at room temperature. The pellet was resuspended in 3 ml and further diluted to a concentration of 1 × 10^6 human erythrocytes/ml in PBS buffer. Lyophilized peptides in various concentrations were resolved in 200 µl of PBS buffer, and 50 µl of human erythrocytes were added following incubation under gentle shaking at 37°C for 1 h. The samples were then placed on ice and centrifuged (4°C). The supernatant was removed, and the pellet was resuspended in 240 µl of water. Release of hemoglobin was monitored by measuring the absorbance of supernatant and water-treated pellet at 541 nm in a 1-cm cell (Jasco V-550). The negative control (0% hemolysis) was 50 µl of human erythrocytes in 200 µl of PBS buffer, and the positive control (100% hemolysis) was 50 µl of human erythrocytes in 200 µl of water. The concentrations of peptides at which 50% hemolysis was observed (EC50) were derived using a sigmoidal curve fitting software (GraphPad Prism 3.0, GraphPad Software).

Bioassays—Bioassays using Drosophila melanogaster according to Escoubas et al. (31) were performed to estimate the LD50 (24 h postinjection) of the peptides. For each assay 20 flies were used as control (injecting 0.05 µl of insect reaper) and 20 for each of the three peptide concentrations. LD50 stands for the lethal dose (50% of the test flies die of intoxication) and calculations were done as described elsewhere (32).

RESULTS

Purification of Cupiennins—450 µl of venom was separated in a five-step protocol that included gel filtration (Fig. 1A), cationic exchange chromatography (Fig. 1B), and successive reversed-phase-HPLC on a nucleosil 100-5 C4 column (not shown), a nucleosil 120-5 C4 column (Fig. 1C) and a nucleosil 100-5 C8 column (Fig. 1D). The retention times of the purified antimicrobial peptides ranged from 18.35 min for cupiennin 1a to 20.12 min for cupiennin 1d. The retention times revealed no other impurities. The purity of the peptides obtained was additionally examined by ESI-MS, N-terminal sequence analysis, and amino acid composition. The yield of cupiennin 1a (ESI-MS 3798.63 ± 0.51 Da, theoretically 3798.59 Da) was 4.7 µg/µl fractionated venom. This implies that the toxin con-
Fig. 1. Isolation of cupiennins from the venom of the spider C. salei. A, crude venom was first separated by gel filtration on a Superdex 75 column, and the obtained antimicrobial fractions were pooled. B, further separation of the pooled fraction was achieved by cationic exchange on a Mono S HR column. C, using RP-HPLC on a nucleosil 120-5 C18 column, the cupiennins were isolated as a broad peak. D, in a last purification step, using RP-HPLC on a nucleosil 100-5 C8, HD column cupiennin 1a, b, c, and d (1 nmol) were isolated as described under “Experimental Procedures.” Synthesized cupiennin 1a* (1 nmol, not amidated) differed only slightly in retention time. The arrows show small peaks of Met-sulfoxidated cupiennins. Absorbance was measured in milli-absorbance units. RT, retention time.

Sequence Analysis of Cupiennin 1a—Sequence analysis of cupiennin 1a ceased at position 34. To obtain the rest of the sequence, cupiennin 1a was cleaved with chymotrypsin and the peptides generated were separated by RP-HPLC on a nucleosil 120-5 C18 column (not shown). The five peaks received were identified by ESI-MS, and the C-terminal peptide (residues 29–35, ESI-MS 421.28 Da, theoretically 421.09 Da) thus also confirmed the presence of methionine sulfoxide at position 34. The oxidation of methionine is a well known artifact during the sequencing process. The correct sequence of cupiennin 1a* was confirmed by amino acid analysis, ESI-MS, and Edman degradation (not shown). The determined amino acid sequence of cupiennin 1a and 1a* agree well with the results of the amino acid composition analyses (Table I).

Sequence Analysis of Cupiennin 1b, 1c, and 1d—Comparative amino acid analysis of cupiennin 1a, 1a*, 1b, 1c, and 1d (Table I) revealed only slight differences in the content of Ala, Ser, Glx, Ile, Val, His, Thr, and Met. Therefore the amino acid sequence determination of cupiennin 1b, 1c, and 1d was performed by comparative trypptic peptide mapping. Peptide separation was carried out by RP-HPLC (Fig. 3, A and B), and the peptides obtained were then identified by ESI-MS. The trypptic peptides of cupiennin 1a and 1a* are identical (Table II) except for the C-terminal peptides, which are amidated in the case of cupiennin 1a, as indicated by the mass difference of 0.85 Da (ESI-MS). Cupiennin 1b differs from cupiennin 1a: 1) in the N-terminal peptide 1–7, where Ala-4 is exchanged with Ser; and 2) in the peptide sequence 28–32, where Val-29 and Val-30 are replaced by Ile-29 and Ala-30. However, cupiennin 1c and 1d also show the single replacement of Ser at position 4 in peptide 1–7. The sequence 28–32 of cupiennin 1c is identical to fragment 28–32 of cupiennin 1b (Val-29 and Val-30 substituted by Ile-29 and Ala-30), but in addition Met-34 is replaced by Thr in the C-terminal peptide 33–35. Cupiennin 1d differs in the C-terminal region from cupiennin 1a in fragment 28–32, where Val-30 is replaced by Ala, and in fragment 33–35, where Gln-33 is exchanged with His (Fig. 2B). The sequences of all trypptic peptides that differed from cupiennin 1a fragments were later determined by Edman degradation (Table II). The deduced amino acid sequences of cupiennin 1b, 1c, and 1d (Fig. 2B) are in good agreement with the results of the amino acid analyses (Table I). For further studies a cupiennin 1d analogue with Gln-33 (ESI-MS 3794.90 ± 0.44 Da, theoretically 3795.55 Da) was also synthesized as nonamidated C terminus (cupiennin 1d*).

General Structural Features—Cupiennin 1a, 1b, 1c, and 1d are linear peptides consisting of 35 amino acid residues. The C terminus is amidated, and the net positive charge is at least +8 at neutral pH (Fig. 2B). The theoretical isoelectric point is 11.30 for all peptides, not taking the C-terminal amidation into account. The N-terminal part of the sequences (Gly-Phe-Gly-
Amino acid sequences of cupiennins from the venom of the spider C. salei. A, amino acid sequence of cupiennin 1a acquired by sequence analysis until position Met-34 and the chymotryptic peptide 29–35. B, overview of amino acid sequences of cupiennin 1a*, 1a, 1b, 1c, 1d, and 1d* deduced from a combination of tryptic peptide mapping and sequence analysis of nonidentical tryptic peptides.

The amino acid composition of cupiennin 1a, 1b, 1c, and 1d from C. salei venom purified by RP-HPLC and synthesized cupiennin 1a* and 1d*.

The values in parentheses are those calculated from the amino acid sequence. ND, not determined.

| Amino acid | Cupiennin 1a | Cupiennin 1a* | Cupiennin 1b | Cupiennin 1c | Cupiennin 1d | Cupiennin 1d* |
|------------|--------------|---------------|--------------|--------------|--------------|--------------|
| A          | 0.8 (1)      | 0.9 (1)       | 0.9 (1)      | 0.8 (1)      | 0.8 (1)      | 0.9 (1)      |
| B          | 3.8 (4)      | 4.0 (4)       | 3.9 (4)      | 3.7 (4)      | 2.9 (3)      | 3.0 (3)      |
| Ser        | 0.1 (0)      | 0.0 (0)       | 1.0 (1)      | 0.9 (1)      | 0.9 (1)      | 0.8 (1)      |
| Gly        | 2.0 (3)      | 3.1 (3)       | 3.0 (3)      | 2.9 (3)      | 2.9 (3)      | 2.9 (3)      |
| His        | 0.0 (0)      | 0.0 (0)       | 0.0 (0)      | 0.0 (0)      | 0.8 (1)      | 1.0 (1)      |
| Arg        | 0.0 (0)      | 0.0 (0)       | 0.1 (0)      | 0.0 (0)      | 0.0 (0)      | 0.0 (0)      |
| Thr        | 0.9 (1)      | 1.0 (1)       | 1.0 (1)      | 1.8 (2)      | 1.0 (1)      | 1.0 (1)      |
| Ala        | 7.0 (7)      | 7.5 (7)       | 6.5 (7)      | 6.5 (7)      | 6.7 (7)      | 7.4 (7)      |
| Pro        | 0.0 (0)      | 0.0 (0)       | 0.1 (0)      | 0.0 (0)      | 0.0 (0)      | 0.0 (0)      |
| Tyr        | 1.0 (1)      | 1.0 (1)       | 1.0 (1)      | 1.1 (1)      | 1.1 (1)      | 1.0 (1)      |
| Val        | 3.3 (4)      | 3.3 (4)       | 2.0 (2)      | 2.0 (2)      | 2.9 (3)      | 3.0 (3)      |
| Met        | 0.7 (1)      | 1.0 (1)       | 0.6 (1)      | 0.1 (0)      | 0.6 (1)      | 0.9 (1)      |
| Cys        | 0.0 (0)      | 0.0 (0)       | 0.0 (0)      | 0.9 (1)      | 0.1 (0)      | 0.0 (0)      |
| lle        | 0.0 (0)      | 0.0 (0)       | 0.0 (0)      | 0.9 (1)      | 0.9 (1)      | 0.0 (0)      |
| Leu        | 2.0 (2)      | 2.1 (2)       | 2.1 (2)      | 2.0 (2)      | 2.0 (2)      | 2.1 (2)      |
| Phe        | 3.0 (3)      | 3.2 (3)       | 2.8 (3)      | 2.9 (3)      | 2.9 (3)      | 3.0 (3)      |
| Lys        | 7.6 (8)      | 7.8 (8)       | 7.4 (8)      | 7.4 (8)      | 7.5 (8)      | 8.0 (8)      |
| Trp        | ND (0)       | ND (0)        | ND (0)       | ND (0)       | ND (0)       | ND (0)       |
| Total      | 33.1 (35)    | 34.9 (35)     | 33.3 (35)    | 33.0 (35)    | 33.1 (35)    | 35.0 (35)    |

Ala/Ser-Leu-Phe) is somewhat hydrophobic, whereas polar amino acid residues predominate in the C-terminal region. All cupiennins are characterized by six repeats of four amino acids, which form the central part of the peptide chain, following the N-terminal hydrophobic stretch with lysine at every first position in their central part. These six repeats are defined by the following sequence: position 1 is always lysine; position 2 is variable (hydrophobic, charged, or polar amino acid); position 3 is always a hydrophobic amino acid (Leu, Val, Ala, Ile) or Gly; and in position 4 is Ala or Val (Fig. 2B). Based on the consensus scale of hydrophobicity for the individual amino acid residues of Eisenberg (33) the mean hydrophobicity of the (H) of the cupiennins was found to range between −0.138 and −0.168. Assuming an α-helical conformation, their hydrophobic moment (μ) was determined to vary between 0.0121 and 0.0282 (Fig. 4A, Table III). These parameters differ distinctly from H and μ values characterizing other antimicrobial peptides such as lyctocoxins (24) isolated from spider venom, melittin (34) from bee venom, and magainin2 (35) found in frog skin. However, the mean hydrophobicity as well as the hydrophobic moment of the cupiennins are distinctly lower than the H and μ values (H = −0.86, μ = 0.2244), magainin 2 (H = −0.036, μ = 0.2861), and lyctocoxin I (H = −0.083, μ = 0.0681) (Table III). The angle subtended by polar residues (Φ) describing the hydrophilic helix surface is at 220° unambiguously greater than the polar face of most other helical antimicrobial peptides (Fig. 4A). The amphipathic motif becomes obvious in the helix net projection. The surface of the cupiennin helix is characterized by a right-handed ribbon of positively charged (lysine) side groups and polar amino acids winding around the α-helix (Fig. 4B).

Circular Dichroism Spectroscopy—The CD spectra of the peptides were recorded in sodium phosphate buffer and exhibit an unordered peptide structure (Fig. 5a). The addition of TFE induced pronounced spectral changes (Fig. 5, b and c). The negative bands at 207 and 222 nm and the positive ellipticity below 200 nm are characteristic of an α-helical conformation. Therefore all peptides were found to be completely helical in the TFE/buffer (1/1 v/v) mixture (Fig. 5). Following Lehrman et al. (36), who suggested that TFE-induced helicity of peptides is a measure of their helix propensity, we take the high a of the investigated cupiennins as an indication of their very high capacity to assume a helical conformation (Table III).

Antibacterial Effects—The cupiennins are highly active against bacteria. Interestingly all four tested bacteria species were susceptible to cupiennin 1a, 1a*, 1d, and 1d* in the nanomolar to micromolar concentration range (Table IV). No clear differences in the minimal inhibitory concentrations were observed between the amidated natural or C-terminal free synthesized cupiennins and E. coli (0.31–0.63 μmol/L; cupiennin 1a and 1a*) and E. faecalis (2.5–5.0 μmol/L cupiennin 1a and 1a*; 1.25–2.5 μmol/L cupiennin 1d and 1d*). The activity of cupiennin 1a (more active) and cupiennin 1a* against P. aeruginosa and S. aureus and of cupiennin 1d and 1d* against P. aeruginosa and S. aureus differed by only one dilution step, whereas the bactericidal effect of cupiennin 1d (more active) and 1d* against E.
coli differed by two dilution steps. Pronounced differences in the susceptibility of Gram-positive and Gram-negative bacteria could not be found. *E. faecalis* exhibited the weakest susceptibility against the cupiennins. Here, melittin was one dilution step more active. However, in the same test system magainin 2 showed no growth inhibition of the tested bacteria up to a...
Insecticidal Effects—We also investigated insecticidal effects in a bioassay with \textit{D. melanogaster}. Surprisingly, cupiennin 1a, 1a*, 1b, 1d, and 1d* showed LD\textsubscript{50} concentrations between 4.7 and 7.9 pmol/mg fly measured after 24 h (Table V). Differences between the LD\textsubscript{50} doses of the synthesized cupiennins 1a\textsuperscript{*} and 1d\textsuperscript{*} and the natural forms were marginal. Obviously, the neurotoxic effects are independent of C-terminal amidation. In comparison with melittin (14.6 pmol/mg fly) the toxicity of the cupiennins was 2.3–3.1 times higher, and compared with magainin 2 (123.1 pmol/mg fly), it was 3.1 times higher, as cupiennin 1a and cupiennin 1d (30%). Magainin 2 showed no hemolytic effect.


data table

TABLE III

| Peptide        | Molecular mass (Da) | Net charge | Mean hydrophobicity (H) | Mean hydrophobic moment (\(\mu\)) | \(\alpha\) (%) |
|----------------|---------------------|------------|-------------------------|-----------------------------------|---------------|
| Cupiennin 1a   | 3798.59             | +8         | –0.138                  | 0.0226                            | 100           |
| Cupiennin 1a*  | 3799.58             | +7         | –0.138                  | 0.0226                            | 100           |
| Cupiennin 1b   | 3800.57             | +8         | –0.155                  | 0.0229                            | ND            |
| Cupiennin 1c   | 3770.48             | +8         | –0.168                  | 0.0121                            | ND            |
| Cupiennin 1d   | 3795.55             | +8         | –0.152                  | 0.0282                            | ND            |
| Cupiennin 1d*  | 3795.55             | +8         | –0.152                  | 0.0282                            | 100           |
| Magainin 2*    | 2466.93             | +3         | –0.038                  | 0.2861                            | 57\textsuperscript{b} |
| Melittin       | 2846.51             | +6         | –0.086                  | 0.2244                            | ND            |
| Lycotoxin I    | 2943.50             | +6         | –0.083                  | 0.0881                            | ND            |
| Lycotoxin II*  | 3206.94             | +6         | –0.219                  | 0.1267                            | ND            |

\textsuperscript{a} The molecular masses were determined using the ExPASy-PeptideMass program as average [M] (www.expasy.ch).
\textsuperscript{b} Data from Ref. 37.

\textsuperscript{c} L. Kuhn-Nentwig, unpublished results.
of cupiennin 1a, 1b, and 1c, and 29.7% identical with that of cupiennin 1d. Thus, the structural peculiarities of cupiennins appear to be unique.

According to the secondary structure prediction method of Garnier et al. (46), all cupiennins show a high probability of forming an a-helix. The enhanced helicity of cupiennin 1b, 1c, and 1d can be associated with the replacement of Val-29—Val-30 in cupiennin 1a by residues of higher helix propensity (Val-29—Ala-30 [cupperninn 1d]; Ile-29—Ala-30 [cupiennin 1b and 1c]) (47). CD measurements in the presence of 30 and 50% structure-inducing TFE confirm the high helix forming potential of the peptides.

Many antimicrobial and hemolytic peptides are able to form highly amphipathic helices, and their structural motifs have been described as effective modulators of activity (48). Thus, the magainin helix is characterized by a polar face dominated by the cationic residues that are spread over the whole peptide chain and a hydrophobic surface. H and \( \mu \) are moderate (Table III). In contrast to magainin, the melittin helix is determined by the extensive hydrophobic N-terminal peptide domain, whereas the C-terminal part of the helix cylinder is highly cationic (48). The helical wheel projection of cupiennin 1a (Fig. 4A) did not show a distinct separation of polar and hydrophobic surface domains. However, pronounced clustering became obvious in the helix net projection (Fig. 4B). The surface of the cupiennin helix is characterized by a charged spiral shaped ribbon of at least six lysine residues and polar groups that connects the more hydrophobic N-terminal with the polar C-terminal domain. This spiral motif of lysine residues determining the helix surface of the cupiennins is to our knowledge unique for antimicrobial peptides from arthropods.

**Bactericidal and Hemolytic Activities**—A comparison of the MIC values reported here with results obtained by other authors is rather difficult, because different assay conditions (plate versus liquid growth assay) and bacterial strains were used. For reasons of standardization ATCC strains were used, and the MIC experiments were performed as reported by Wu and Hancock (30). The minimal inhibitory concentrations of the most active cationic antimicrobial peptides have been reported to range between 0.25 and 4 \( \mu \)g/ml (2). The tested cupiennins are highly active against both Gram-negative and Gram-positive bacteria as shown by MICs (\( [\text{a}] \)) in Table IV) between 0.3 \( \mu \)g/ml for cupiennin 1d against *E. coli* and up to 9.5 \( \mu \)g/ml for cupiennin 1a/1a* against *E. faecalis*. The MIC range of cupiennins contains six dilution steps in contrast to three in the case of melittin. This may indicate differences in the mode of action. Studies of a variety of natural peptides and their chemically
modified analogs demonstrate that the cationic charge is essential for recognition and accumulation on the negatively charged bacterial membranes (2, 49, 50). With their high content of positively charged lysine residues, the cupiennins fulfill this prerequisite. To possess antimicrobial activity, less hydrophobic peptides should have a well developed amphipathic helix and high peptide hydrophobicity mediate the hemolytic effect (37, 51, 52). The activity is determined by hydrophobic interactions between the nonpolar amino acid residues and the hydrophobic core of the lipid matrix of red blood cells (53). The cupiennins are composed of 46–49% hydrophobic amino acids and show high helicity in the presence of TFE, but structural motifs favoring hydrophobic interactions such as high hydrophobicity and a pronounced hydrophobic moment are poorly developed. H and \( \mu \) values are much lower in the cupiennins than in the highly hemolytic melittin and even lower than in the nonhemolytic magainin 2. Thus, comparable with melittin, insertion of the hydrophobic N-terminal helix domain into the hydrophobic core of the neutral lipid matrix of red blood cells seems to be responsible for the hemolytic effects of the cupiennins. Additional ionic interactions between the negatively charged \( O \)-glycosidic chains of glycoporphin A of erythrocytes (54) and the highly cationic lysine ribbon of the cupiennin peptides might support membrane insertion followed by bilayer disruption, pore formation or lysis. The hypothesis is supported by Helmerhorst et al. (55), who suggested that electrostatic interactions govern the peptide-erythrocyte interaction.

In summary, the cupiennins have been found to be a class of antimicrobial, hemolytic, and bactericidal peptides with unique structural properties. Additional studies are in progress to further elucidate the structural basis of the diversity in their biological activity. Furthermore, the peptides appear to be available as model compounds for the investigation of the structural background of membrane lysis and selectivity to different target membranes.

Acknowledgments—We thank U. Kämper, H. Petrenko, and S. Luethi for their excellent technical assistance, Dr. T. Bodmer for kindly providing the bacteria, and Dr. H. Murray and Dr. C. Boesel for helpful discussion.

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