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Permeabilization of the mitochondrial inner membrane by short cecropin-A–melittin hybrid peptides

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A number of cecropin-A−melittin hybrid peptides have previously been shown to be potent antibacterial agents (Andreu, D., Ubach, J., Boman, A., Wahlin, B., Wade, D., Merrifield, R. B. & Boman, H. G. (1992) *FEBS Lett. 296*, 190−194). In the present report we analyze their action on biological systems using rat liver mitochondria as a test system. We demonstrate that the longest peptide, cecropin-A-(1−8)−melittin(1−18) permeabilizes the mitochondrial inner membrane allowing the movement of both charged and non-charged solutes. Concentrations used have already been shown to be bactericidal. This effect is also demonstrated under respiring conditions where succinate oxidation is uncoupled. Shorter analogs also permeabilize mitochondria although at ten-fold higher concentrations. Heparin potentiates the peptide effects at low concentrations, while at high concentration it becomes inhibitory. We propose that the cecropin-melittin analogs disrupt the mitochondrial membrane in a detergent-like mode rather than by creating selective channels as had been previously suggested.

Antibacterial peptides are one of the main components of the non-specific immunity in pluricellular organisms. They have been described in insect hemolymph, intestinal and tracheal mucosas, frog skin secretions, neutrophils, macrophages, etc. They are grouped in different families according to their similarity in primary structure (Boman et al., 1991; Zasloff, 1992; Hoffman et al., 1993; Lehrer et al., 1993).

Cecropins are one of the main families of antibacterial peptides which are present in insect hemolymph (reviewed in Boman et al., 1991). They are polypeptides of less than 40 residues, lacking disulfide bonds. Three regions can be distinguished: the N-terminal end, with several positively charged lysine residues; a central hinge region formed by a Pro-Gly sequence; and finally a C-terminal hydrophobic stretch. Therefore when the protein is in a non-polar environment it adopts an «-helix−hinge−«-helix conformation (Holak et al., 1988). There appear not to be specific receptors for cecropins in the bacterial membrane (Wade et al., 1990); the peptides adsorb to the bilayer and aggregate to form voltage-dependent channels which collapse the ionic gradients (Christensen et al., 1988; Merrifield et al., 1991). These structural features are also shared by certain peptides present in insect venoms. Thus, melittin, one of the main components of bee venom, has a lytic action both against bacteria and eukaryotic cells. When compared with cecropins, the sequence orientation is in the reverse order, i.e. the C-terminal end is the positively charged one. The mechanism of action is still controversial (reviewed in Dempsey, 1990; Sansoin, 1991).

Since cecropins are relatively short peptides, chemical synthesis has been employed for the study of structure/function relationships. Thus it has been used for the definition of the role of specific residues and to improve their antibiotic activity (Andreu et al., 1985; Steiner et al., 1988; Fink et al., 1989). The possible clinical application of antibacterial peptides requires first the lack of harmful effects on the patient (peptide immunogenicity, lytic effects, etc.) and second a low cost of synthesis. Boman et al. (1989) and Andreu et al. (1992) have concentrated on the design of cecropin-A−melittin hybrids as antibacterial peptides with broad specificity. These peptides are composed, from the N-terminus to the C-terminus, of the first eight amino acids of the N-terminal sequence of cecropin A followed by a variable stretch of amino acids from the N-terminus of melittin. The elimination of the C-terminal end from the melittin molecule greatly diminishes its hemolytic activity. Some of the active peptides of these series have a sequence shorter than that required to span the thickness of the bilayer; thus, their mode of action must differ from that of the parental peptides (Merrifield et al., 1991; Andreu et al., 1992).

In the present study we intend to set a protocol for testing the activity of these chimeric peptides and thus help in the design of new antibiotic peptides. We have used mitochondria as our *in vitro* system for looking at the effect that these analogs have on biological membranes. Other bactericidal peptides, such as magainins (Westerboff et al., 1989a and b; Juretic et al., 1989), cecropins and some derivatives have been tested as uncoupling agents in mitochondria, as well as competitors in the import of precursors proteins into mitochondria (Hugosson et al., 1992). We conclude that the cecropin-A−melittin hybrids tested cause a non-specific per-

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Abbreviations. FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; LPS, lipopolysaccharide; MTP, mitochondrial-membrane-permeability transition pore; Boc, butoxycarbonyl.

meabilization of the mitochondrial inner membrane. We postulate that they perturb the lipid bilayer in a detergent-like fashion rather than inducing ion-specific channels.

MATERIALS AND METHODS

Materials

/V'-Butoxycarbonyl(Boc)-protected amino acid derivatives were obtained from Novabiochem and Propeptide. All other biochemicals were from Sigma or Merck.

Peptide synthesis

Peptides were synthesized by the solid-phase method (Merrifield, 1986) as previously described (Andreu et al., 1992) using Boc-chemistry protocols on /ɹ-methylbenzhydrylamine resin. The peptides were deprotected and cleaved from the resin by HF acidolysis and purified by reverse-phase chromatography on a CIS column. us described. The sequences of the hybrid peptides used for this study are shown in Table 1. All analogs had an amidated carboxyl group.

Isolation of mitochondria and determination of permeability properties

Rat liver mitochondria were isolated as previously described (Heaton and Nicholls, 1976). Final pellet was resuspended in a buffer containing 250 mM sucrose, 5 mg/ml albumin, 10 mM Tes, pH 7.4. Protein concentration was determined by the Biuret method using albumin as standard.

The respiration experiments were carried out at 25°C in a Hansa-Tech O$_2$ electrode chamber. Mitochondria (1 mg/inl) were suspended in medium containing 75 mM NaCl. Tes 10 mM, EGTA 1 mM. 2 tng/ml albumin. 2 mM sodium phosphate, pH 7.0. Substrate was either 5 mM succinate. or 5 mM glutamate plus 5 mM malaie.

Mitochondrial permeability to sucrose. potassium acetate or sodium chloride was determined from the rate of absorbance decrease (550 nm) due to changes in light scattering during osmotic swelling. When control mitochondria were suspended in 100 mM potassium acetate. 20 mM Tes. 1 mg/nil albumin. 5 JIM rotenone. pH 7.0. the permeability to either protons (valinomycin 0.5 JIM present) or to potassium |10 ɑiM carbonylcyanide /ɔ-trifluoromethoxyphenylhydrazone (FCCPi present] was determined (data not shown). However. since peptide addition stimulated both permeabilities simultaneously no ionophores were needed in routine experiments. Permeability to sucrose was determined in medium containing 250 mM sucrose. 10 mM Tes. 1 mg/ml albumin. pH 7.0. Permeability to NaCl was determined with mitochondria suspended in the buffer used in the respiration experiments. In all cases, protein concentration was 0.25 mg/ml and incubation temperature 25°C.

RESULTS

Alteration of the permeability properties of the mitochondria inner membrane by the peptide cecropin-A-(1–8)-melittin(1–18)-NH$_2$

Cecropin and melittin have been shown to form channels and permeabilize biological membranes as part of their mechanism of action (Christensen et al., 1988; Wade et al., 1990; Dempsey et al., 1990). The synthesis of cecropin-melittin hybrid peptides has proven to be a useful approach for the design of more potent antibacterial peptides with broader specificity against pathogens while avoiding the toxic effects on eukaryotic cell tvpes (Andreu et al., 1992; Boman et al., 1989).

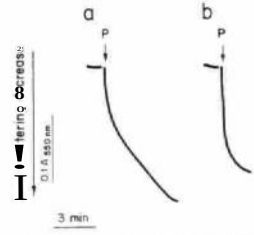


Fig. 1. Effect of 4 nmol/mg CA(1–8)M(1–18)-NH$_2$ on the permeability to sucrose (a) and potassium acetate (b) of rat liver mitochondria. Permeabilities were determined from the rate of light-scattering decrease during osmotic swelling when mitochondria were suspended either in 250 mM sucrose or 100 mM potassium acetate. See Materials and Methods for further details. Peptide was added when indicated. P.

We have set out to test the effects of four such hybrid peptides, with two goals: first, to advance in the understanding of their mechanism of action, and, second to develop tests for fast screening of newly developed peptides. We have used isolated rat liver mitochondria as a model system to study the interaction of the cecropin-melittin hybrids with biological membranes. This system has interesting advantages since plenty of material can be obtained easily with very reproducible characteristics, and there are techniques that allow a detailed investigation of their permeability properties.

We have used a technique based on the osmotic swelling of the mitochondrial matrix to investigate the variations in the permeability properties of the inner membrane brought about by the action of the cecropin-melittin hybrids. It has been demonstrated that changes in light scattering resulting from changes in mitochondrial volume can be applied for the quantitative determination of solute transport across the mitochondrial inner membrane (Beavis et al., 1985; Carlid and Beavis, 1985). Since net import of solutes is accompanied by the entry of water and this results in mitochondrial swelling, it is possible to determine the rate of solute entry from the rate of light-scattering decrease.

The cecropin-melittin hybrid peptide cecropin-A-(1–8)-melitlin-cl – 18)-NH$_2$ [CA(1–8)M(1–18)-NH$_2$] is composed of the first 8 residues of cecropin A followed by the first 18 residues from melittin (Table 1 and Andreu et al., 1992). The peptide has a net positive charge at physiological pH due to the presence of five lysines and displays a high «-helical content in *4%* hexafluoroisopropanol (Wade et al., 1990). Its antibiotic and lytic activity is higher than those of cecropin A and melittin while it has no effect on sheep erythrocytes (Wade el al., 1990; Andreu et al., 1992).

The effect of the peptide CA(1–8)M(1–18)-NH$_2$ on the permeability properties of the inner membrane is shown in Fig. 1. It induces swelling both in sucrose and potassium acetate. The swelling traces also reveal different kinetics; while the peptide action in acetate is fast and almost complete within 60 s, swelling in sucrose is slower although it pro-

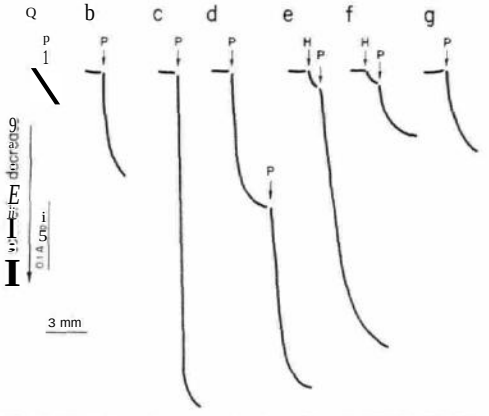


Fig. 2. Effect of varying concentrations of CA(1–8)M(1–18)-NH_2 and heparin on the swelling of mitochondria in potassium acetate. (a) 2 nmol peptide/mg mitochondrial protein; (b) 4 nmol/mg; (c) 8 nmol/mg; (d) two consecutive additions of 4 nmol/mg; (e) heparin 0.13 mg/ml plus 4 nmol peptide/mg mitochondrial protein; (f) heparin 0.65 mg/ml plus 4 nmol peptide/mg; (g) addition of a previously incubated mixture of 4 nmol peptide/mg plus 0.13 mg heparin/ml. Additions: H, heparin; P, peptide CA(1–8)M(1–18)-NH_2.

ceeds to a greater extent. At this stage it is very difficult to ascertain the reason for these differences although it is very likely that the interaction of the peptide with the membrane will greatly depend on the ionic strength and this is very different in the two media. The concentration chosen (1 μM, i.e. 4 nmol/mg protein) has been shown to be bactericidal for most of the species tested (Andreu et al., 1992; Wade et al., 1990).

Mitochondrial swelling in sucrose involves the entry of a polar non-charged solute. Swelling in potassium acetate requires permeation of acetic acid through the bilayer, electrogenic entry of potassium and, in order to maintain charge and pH balance, the exit of one proton. Therefore these results show that while in sucrose the peptide CA(1–8)M(1–18)-NH_2 has created a path for the movement of a non-charged molecule, in the second case it allows the simultaneous movement of potassium in one direction and protons in the opposite one. Previous reports have indicated that permeabilization of planar membranes by cecropin A proceeds after the formation of voltage-sensitive channels with a permeability ratio Cl^-/Na^+ of 2:1 (Christensen et al., 1988; Wade et al., 1990). However, these swelling experiments reveal that permeation occurs in the absence of membrane potential and that both charged and non-charged species can cross the membrane.

The pathway induced by the peptide is reminiscent of the mitochondrial-permeability-transition pore (MTPi since the MTP allows permeation of a wide variety of small molecules, including sucrose. MTP opening can be induced by many factors which do not share common structural or functional features and it is inhibited with high specificity by cyclosporin A (Gunter and Pfeiffer, 1990; Petronilli et al., 1993). To test the possibility that the peptide-induced permeability is due to MTP opening, experiments were performed where the ability of cyclosporin A to inhibit the peptide-induced swelling was tested. Cyclosporin A (up to 20 pM) did not affect either sucrose or acetate swelling induced by CA(1–8)M(1–18)-NH_2 (data not shown).

Fig. 2 shows the effect of varying concentrations of added CA(1–8)M(1–18)-NH_2. It is apparent that the peptide associates with the membranes so fast that it does not fully equilibrate with the medium. Thus at low peptide concentrations only a limited population of mitochondria will reach

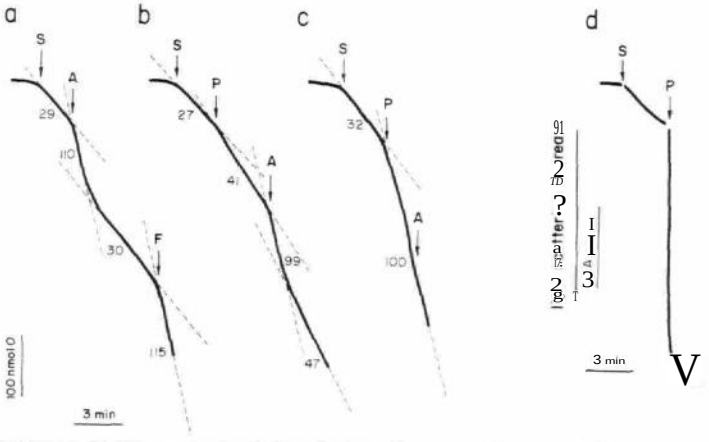


Fig. 3. Effect of CA(1–8)M(1–18)-NH_2 on mitochondrial respiration with succinate as substrate. (a) Control mitochondria; (b) effect of 1 nmol pepude/mg on the respiratory control; (c) effect of 3 nmol pepride/mg; (d) induction of mitochondrial swelling by 4 nmol peptide/mg under the conditions used in respiration experiments (NaCl buffer). See Materials and Methods for further details. Additions: S, 5 mM succinate; A, 0.2 mM ADP; F, 10 pM FCCP; P, peptide CA(1–8)M(1–18)-NH_2.

the critical level of peptide in their membrane to allow permeabilization. and a second addition allows new mitochondria to reach this critical level. This fast association can be modulated if a polyanion is present. Fig. 2. trace e. shows the effect of 4 nmol/mg CA(1−8)M(1−18)-NH_2 when added to the mitochondrial suspension in the presence of heparin 0.13 mg/ml. The kinetics reveal a slower permeabilization process but reaching to the entire mitochondrial population. thus similar to the effects observed with low ionic-strength medium such as sucrose (Fig. 1, trace a). Higher heparin concentrations (0.65 mg/ml) become inhibitory (Fig. 2. trace f). When the heparin and the peptide are allowed to equilibrate in the incubation mixture and then the mix is added to mitochondria. the action of the peptide is significantly reduced (Fig. 2. trace g).

Effect of CA(1–8)M(1–18)-NH_2 on mitochondria under respiring conditions

If the interaction of the antibacterial peptides were to be dependent on a membrane potential. then the conditions closest to the *in vivo* situation would be those under respiring conditions. In fact. the presence of a membrane potential should favor insertion of the positively charged peptide in the membrane. Fig. 3 shows the effect of CA(1−8)M(1−18)-NH_2 on mitochondria oxidizing succinate. Addition of 1 nmol peptide/mg mitochondrial protein stimulates respiration 1.5-times. However. subsequent addition of ADP still allows observation of respiratory control, with a 3.6-fold stimulation of respiration. With CA(1−8)M(1−18)-NH_2 at 3 nmol/mg. respiration is stimulated threefold. but respiratory control by ADP is completely lost (Fig. 3. trace c). Therefore it appears that the peptide is acting as an uncoupling agent. similarly to magainins fWesterhoff et al., 1989a and b). However. when mitochondrial swelling is monitored under respiration conditions (buffer has NaCl as osmotic support). an extremely fast peptide-induced swelling is observed (Fig. 3. trace d) which suggests that the uncoupling-like effects could be due to the unspecific permeabilization of the inner membrane.

The situation is somewhat different when the mitochondria are respiring with glutamate/malate (Fig. 4). Addition of 3 nmol peptide/mg leads to a transient increase in respiration which decreases progressively and which cannot be re-activated by .ADP or FCCP (Fig. 4. trace b). However. once the glutamate/malate respiration has been inhibited by the peptide. the subsequent addition of succinate increases markedly the rate of respiration. although it is not sensitive to ADP or FCCP (Fig. 4. trace c). Since peptide addition leads to permeabilization of the membrane and mitochondrial swelling (Fig. 3, trace d) and hence leakage of solutes from the matrix. it is not surprising that it impairs glutamate/malate oxidation.

The effect of peptide analogs of CA(1–8)M(1–18)-NH_2

One of the aims of the present work was to set a protocol for the characterization of the action of peptide analogs. Table 1 presents the amino acid sequence of three shortened cecropin-A−melittin hybrids. They all consist of the first seven residues of the N-terminal end of ceeropin A plus eight residues corresponding to different parts of the N-terminal sequence of melittin (Andreu et al., 1992). Fig. 5 shows the effect of the addition of 80 nmol peptide/mg on the osmotic swelling of mitochondria in potassium acetate. No substantial

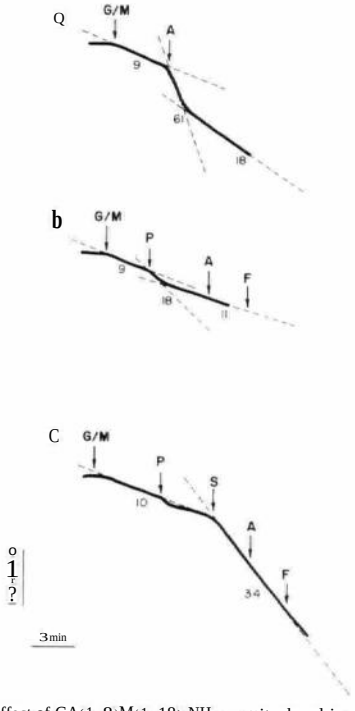


Fig. 4. Effect of CA(1–8)M(1–18)-NH_2 on mitochondria respiring with glutamate and malate as substrate. (a) Control mitochondria; (b) effect of 3 nmol peptide/mg; (c) effect of 5 nmol/mg. Additions: G/M. 5 mM glutamate plus 5 inM malate; A. 0.2 mM ADP: S. 5 mM succinate: F. 10 pM FCCP. P. peptide CA(1−8)M(1−18)-NH_2.

differences are observed among them. However. it should he noted that when compared with the peptide CA(1−8)M(1−181-NHj. higher concentrations are required to permeabilize mitochondria and they only induce low-amplitude swelling. When heparin is present. the effects are now similar to those of CA(1−8)M(1−18)-NH_2 although high peptide concentrations are still required.

DISCISSION

The proposed mechanism of interaction of cecropins with biological membranes has two sequential steps: first. interaction of the positively charged N-terminal region with the negative components of the external layer of the membrane and. second. insertion of the C-terminal hydrophobic sequence into the bilayer. The channel is formed by aggregation of ceeropin molecules in the membrane displaying single-channel conductance (Christensen ct al., 1988: Wade et al., 1990). Recently. a computer model of ceeropin A channel has been described where two types of channels are considered. Both are based on the aggregation of a basic dimer structure. formed by two anliparallel peptide chains. but while in one case the internal lining of the channel is pro-

Table 1. Amino acid sequence. α-helix content and antibacterial effect of the cecropin-A–melittin hybrid peptides. Sequences are divided in two regions, the N-terminal end belongs to cecropin A and the C-terminal end to melittin. The α-helix contents in 89% hexafluoro-2-propanol are taken from Andreu et al. (1992). Lethal concentrations to *Pseudomonas aeruginosa* (Pse) and *Staphylococcus aureus* (Stap) were calculated from inhibition zones on agarose plates; for *Plasmodium falciparum* (Mai) they were calculated from the inhibition of erythrocyte reinvasion, data from Andreu et al. (1992).

Peptide	Amino acid sequence	α-helix content	Lethal concentration to		
			Pse.	*Stap.*	*Mai.*
			μM		
CAM(1–8)M(1–18)-NH_2	KWKLFKKI GIGAVLKVLTTGLPALIS	42[a]	0.7	1	4
CA(1–7)M(2–9)-NH_2	KWKLFKK IGAVLKVL	28	4	2	7.5
CA(1–7)M(4–11)-NH_2	KWKLFKK AVLKVLTT	0	4	8	8
CA(1–7)M(5–12)-NH_2	KWKLFKK VLKVLTTG	35	2	8	9

[a] Data from Wade et al. (1990).

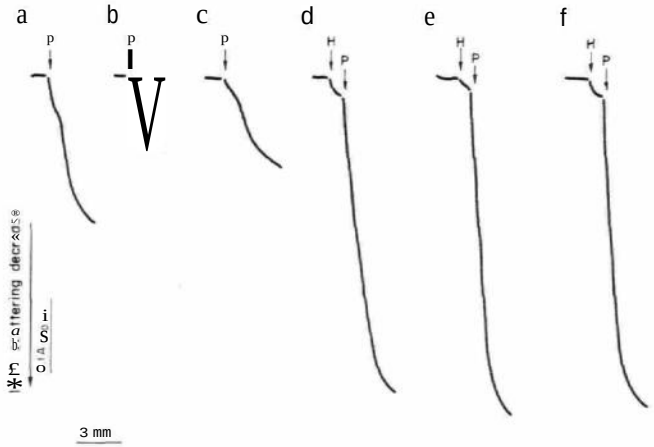


Fig. 5. Effect of various analogs of the peptide CA(1–8)M(1–18)-NH_2 **on the mitochondrial permeability to potassium acetate.** (a and d) CAM(1–7)M(2–9)-NH_2, 80 nmol/mg; (b and e), CA(1–7)M(4–11)-NH_2, 80 nmol/mg; (c and f) CAM(1–7)M(5–12)-NH_2, 80 nmol/mg. (d, e and f) 0.13 mg heparin/ml was added before the peptide. Additions: P, peptide analog; H, heparin.

vided by the N-terminal charged α-helix, in the other case it is the hydrophobic C-terminal α-helix (Durell et al., 1992).

Melittin also forms channels by aggregation of monomers leading to the formation of helix bundles. The orientation will depend on the lipid composition of the bilayer, its hydration level and the presence of a transmembranc potential (reviewed in Dempsey, 1990; Sansom, 1991). The molecular mechanism of action of melittin is far from clear. At low melittin/lipid ratios, peptide aggregation leads to the formation of voltage-gated channels with discrete levels of conductance that will probably depend on the number of oligomers (Hanke et al., 1983; Schwarz et al., 1992). However, at high concentrations, results indicate that it can cause micelle formation from the membranes in a way similar to the solubilization by detergents (Habermann, 1972).

Recently, a number of cecropin-A–melittin hybrid peptides have been developed with the purpose of improving their antibacterial activity while decreasing the undesirable cytotoxic effects (Boman et al., 1989; Wade et al., 1990; Andreu et al., 1992). Their organization in the membrane is not known although their reduced size, absence of both a central Gly-Pro hinge and a Glu9/Arg16 ionic pair (stabilizing the cecropin A dimer) must result in an arrangement different from those suggested for the parental peptides (Durell et al., 1992). In consequence their mechanism of action is also likely to be different. Christensen et al. (1988) have reported that while cecropins form voltage-dependent ion channels, shorter analogs do not form channels although they adsorb to the bilayer.

In the present study we have set out to test the effect of four hybrid peptides on the permeability properties of the mitochondrial inner membrane. We have shown that the interaction of the peptides with the membrane leads to the formation of pores which allow movement of both charged and non-charged molecules. Permeabilization is observed both in the presence and absence of membrane potential. It is interesting that magainins, another family of antibacterial peptides, also induce mitochondrial swelling in sucrose (Westerhoff et al., 1989a). Under respiring conditions, with succinate as substrate, the peptide displays an uncoupling-like beha-

vior. However, since mitochondria swell immediately after the addition of peptide it must be assumed that unspecific permeabilization of the mitochondrial inner membrane has occurred. In our view, the observed lack of specificity of the channels formed suggests that it may be disturbing the bilayer structure rather than creating selective pathways for ion movement.

The method of addition of the peptide does influence the degree of permeabilization. Thus, sequential addition of peptide is less effective than a single dose (Fig. 2, traces c and di. Westerhoff et al. (1989a) also observed differences due to the method of peptide addition with their studies of the interaction of magainins with mitochondria. Since it has been shown that several antibacterial amphipathic peptides can exist as aggregates, our results suggest that this might be a determining factor lor the outcome of the experiment. Preliminary experiments by analytical centrifugation have showm a high degree of aggregation of CA(1−8)M(1−18)-NH_2 at concentrations higher than 20 pM. One of the parental peptides, melittin, presents two states of aggregation in water. At low peptide concentrations it exists mainly as a monomer with a random-coil conformation, while at higher concentrations tetramers are formed and a high «-helix content is detected (reviewed in Dempsey, 1990). The elimination of Pro in a synthetic analog of melittin increases the «-helical content and induces aggregates of more than 50 molecules (John and Jàhnig, 1992). Structural modifications have not been observed with CA(1−8)M(1−18)-NH_2 below 50 (iM, although some loss of helicity can be detected above this concentration (Andreu et al., 1992).

The kinetics of the mitochondria permeabilization suggests a fast association of the peptide aggregate with the membrane. However, the peptide/membrane equilibrium can be modified in the presence of polyanions such as heparin. It should be noted that both heparin and the LPS are negatively charged and that LPS is one of the main components of the membrane playing an important role in the interaction of antibiotic peptides with Gram-negative bacteria (Rana et al., 1991). It is remarkable that at low concentrations there is an enhancing effect of heparin. The quenching of the peptide positive charges by the polyanionic heparin should slow down the peptide adsorption into the negatively charged mitochondrial membranes thus explaining the observed decrease in the rate of permeabilization (Fig. 2, trace e). This would also allow a more homogenous distribution and therefore more mitochondria would reach the threshold level of permeabilization. At higher heparin concentrations, or when peptide and heparin are incubated together before being added to the mitochondrial suspension, a marked inhibition of the peptide effect is observed. While the first case could be explained by simple competitive kinetics, the second case indicates that the formation of the heparin/peptide complex is a slow process. Comparative analysis of CAfl−8)M(1−18)-NH_2 with proteins which bind heparin with high affinity does not reveal any similar motif in this peptide (Margalit et al., 1993). It is possible that the heparin/peptide interaction leads to further alterations in peptide structure.

The effect of heparin could have implications for the cell specificity of the peptides. Magainins and cecropins are known to have lethal effects on lower eukaryotes (Andreu et al., 1992; Boman et al., 1989; Huang et al., 1990; Zasloff et al., 1988) and certain tumor cell lines (Cruciani et al., 1991; Baker et al., 1993) at low concentrations, while showing no effect on erythrocytes or other non-transformed cells. The presence of heparan sulfate and other negatively charged components in the extracellular matrix may be an important factor for peptide specificity and protection.

Andreu et al. (1992) have designed shortened peptides derived from the CA(1−8)M(1−18)-NH_2 which retain antibiotic activity. The working hypothesis for the action of these peptides has been that they lead to the bioenergetic collapse of the bacteria. Having set the conditions with rat liver mitochondria to assess the permeabilization properties of the parental peptide, we tested the effect of the short derivatives and conclude that they are indeed active. However, their mode of action differs from that of CA(1−8)Mi1−18)-$NH_>$ since they require a much higher concentration and the presence of heparin. While long peptides are able to form «-helical structures which would span the membrane and hence could form channels, the action of short peptides would require either the stacking of monomers (Merrifield et al., 1991), adoption of extended (3_{10}) helices (Andreu et al., 1992), or simply the disintegration of the membrane due to a detergent-like action. In the mitochondrial model used in this work, the lack of specificity of the channels formed (swelling is observed both in salts and sucrose) would, in our view, favor this latter possibility. Recently, Schafmeister et al. (1993) have designed a 24-amino-acid peptide which allows solubilization of integral membrane proteins. This peptitergent has «-helical structure with positively charged amino acids at both ends.

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REFERENCES

Andreu, D., Merrifield, R. B., Steiner, H. & Boman, H. G. (1985) N-tenninal analogues of cecropin A: Synthesis, antibacterial activity and conformalional properties. *Biochemistry 24*, 1683−1688.

Andreu, D., Ubach, J., Boman, A., Wahlin, B., Wade, D., Merrifield, R. B. & Boman, H. G. (1992) Shortened cecropin A-melmm hybrids. Significant size reduction retains potent antibiotic activity. *FEES Un. 296*, 190−194.

Baker, M. A., Maloy, W. L., Zasloff, M. & Jacob, L. S. (1993) Anticancer efficacy of magainin 2 and analogue peptides. *Canter Res. 53*, 3052−3057.

Beavis, A. D., Brannan, R. D. & Garlid, K. D. (1985) Swelling and contraction of the mitochondrial matrix. A structural interpretation of the relationship between light scattering and matrix volume. *J. Biol. Client. 260*, 13424−13433.

Boman, H. G., Wade, D., Boman, I. A., Wahlin, B. & Merrifield, R. B. (1989) Antibacterial and antimalurial properties of peptides that are cecropin-melittin hybrids. *FEBS Lett. 259*, 103−106.

Boman, H. G., Faye, I., Gudmundsson, G. H., Lee, J. Y. & Lindholm, D. A. (1991) Cell free immunity in *Cecropin*. A model system for antibacterial proteins. *Eur. J. Biochem. 201*, 23−31.

Christensen, B., Fink, J., Merrifield, R. B. & Mauzerall, D. (1988) Channel-forming properties of cecropins and related model compounds incorporated into planar lipid membranes. *Proc. Natl Acad. Sri. USA* 85, 5072−5076.

Cruciani, R. A., Barker, J. L., Zasloff, M., Chen, H. Q. & Colaniolnici, O. (1991) Antibiotic magainins exert cytolytic activity againsi transformed cell lines through channel formation. *Proc. Natl Acad. Sa. USA* HH, 3792−3796.

Dempsey, C. E. (1990) The actions of melittin on membranes. *Bioeftim. Biophys. Acta 1031*, 143−161.

Durell, S. R., Raghunathan, G. & Guy, H. G. (1992) Modeling the ion channel structure of cecropin. *Biophys J. 63*, 1623−1631.

Fink. J., Merrificld. R. B., Boman. A. & Boman. H. G. (1989) The chemical synthesis of eecropin D and an analog with enhanced antibacterial activity. *J. Biol. Chem. 264*, 6260–6267.
Heaton. G. M. & Nicholls. D. G. (1976) The calcium conductance of the inner membrane and the determination of the calcium electrochemical gradient. *Biochew. J. 156*, 635–646.
Gariid. K. D. & Beavis. A. D. (1985) Swelling and contraction of the mitochondrial matrix. II. Quantitative application of the light scattering technique to solute transport across the inner membrane. *J. Biol. Chem. 260*, 13434–13441.
Gunter. T. E. & Pfeiffer. D. R. (1990) Mechanisms by which mitochondria transport calcium. Aw. *J. Physiol. 258*. C755–C786.
Hanke. W., Methfessel. C., Wilmsen. H. U., Katz. E., Jung. G. *Ik* Boheim. G. (1983) Melittin and a chemically modified trichotoxin form alamethicin-tvpe multi-state pores. *Biochim. Biophw. Acta 943*, 375–380.
Habermann. E. (1972) Bee and wasp venoms. *Science 177*. 314–322.
Hoffman. J. A., Hetru. C. & Reichhart. J. M. (1993) The humoral antibacterial response of *Drosophila*. *FEBS Lett. 325*. 63–66.
Holak. T. A., Engsirom. A., Kraulis. P. J., Lindeberg. G., Bennich. H., Jones. T. A., Gronenbom. A. M. & Clore. G. M. (1988) The solution conformation of the antibacterial peptide eecropin A: A nuclear magnetic resonance and dynamic simulated annealing study. *Biochemistry 27*. 7620–7629.
Huang. C. M., Chen. H.-C. & Zierdt. C. H. (1990) Magainin analogs effective against pathogenic protozoa. *Aniimicrob. Agents Chariother. 34*. 1824–1826.
Hugosson. M., Boman. H. G. & Glaser. E. (1992) in *Molecular, biochemical anil physiological aspects of plant respiration* (Lumbers. H. & van der Plus. L. H. W., edsi pp. 367–372. SPB Academic Publishing. The Hague.
Hugosson. M., Andreu. D., Boman. H. G. & Glaser. E. (1994) Antibacterial peptides and mitochondrial presequences affect mitochondrial coupling. respiration and protein import. *Eur. J. Biochem.*, 1027–1033.
John. E. *ik* Jiihnig. F. (1992) A synthetic analogue of melittin aggregates in large oligomers. *Biophys. J. 63*, 1536–1543.
Juretie. D., Chen. H. C., Brown. J. H., Morell. J. L., Hendler. R. W. & Westerhoff. H. V. (1989) Magainin 2 amide and analogues. Antimicrobial activity, membrane depolarization and susceptibility to proteolysis. *FEBS Lett. 244*. 219–223.
Lehrer. R. I., Lichtenstein. A. K. & Ganz. T. (1993) Defensins. Antimicrobial and cytotoxic peptides of mammalian cells. *Anna. Rev. fmmunol. II*. 105–128.
Margalit. H., Fischer. N. & Ben-Sasson. S. A. (1993) Comparative analysis of structurally defined heparin binding sequences reveals distinct spatial distribution of basic residues. *J. Biol. Chem 268*. 19228–19231.
Merrifield. R. B. (1986) Solid phase synthesis. *Science 232*. 341–347.
Merrifield. R. B., Boman. H. G., Andreu. D., Li. Z.-Q., Fink. J. & Wade. D. (1991) in *Peptides 1990* (Giralt. E. & Andreu. D., eds) pp. 3–16. ESCOM Science Publishers. Leyden.
Merrifield. R. B., Merrifield. E. L., Juvvadi. P., Andreu. D. & Boman. H. G. (1994) Design and synthesis of antibacterial peptides. *CIBA Found Symp 186*. in the press.
Petronilli. V., Cola. C., Massari. S., Colonna. R. & Bemardi. P. (1993) Physiological effectors modify voltage sensing by the cyclosporin A-sensitive permeability transition pore of mitochondria. *J. Biol. Chem. 268*. 21939–21945.
Rana. F. R., Macias. E. A., Sultany. C. M., Modzrakowsky. M. C. & Blazyk. J. (1991) Interactions between magainin 2 and *Salmonella typhimurium* outer membranes: effect of lipopolysaccharide structure. *Biochemistry 30*. 5858–5866.
Sansom. M. S. P. (1991) The biophysics of peptide models of ion channels. *Prog. Biophys. Mol. Biol. 55*. 139–2.35.
Schafmeister. C. E., Miercke. L. J. W. & Stroud. R. M. (1993) Structure at 2.5 Å of a designed peptide that maintain solubility of membrane proteins. *Science 262*. 734–738.
Schwarz. G., Zong. R. T. & Popescu. T. (1992) Kinetics of melittin induced pore formation in the membrane of lipid vesicles. *Biochim. Biophys. Acta II 10*. 97–104.
Steiner. H., Andreu. D. A: Merrifield. R. B. (1988) Binding and action of eecropin and eecropin analogues: antibacterial peptides from insects. *Biochim. Biophys. Acta 939*, 260–266.
Wade. D., Boman. A., Wahlin. B., Drain. C. M., Andreu. D., Boman. H. G. *ik* Merrifield. R. B. (1990) All-D ammo acid-containing channel-forming antibiotic peptides. *Proc. Natl Acad. Sci. USA 87*. 4761–4765.
Westerhoff. H. V., Hendler. R. W., Zasloff. M. & Juretie. D. (1989a) Interactions between a new class of eukaryotic antimicrobial agents and isolated rat liver mitochondria. *Biochim. Biophys. Acta 975*. 361–369.
Westerhoff. H. V., Juretie. D., Hendler. R. W. & Zasloff. M. (1989b) Magainins and the disruption of membrane-linked free-energy transduction. *Proc. Natl Acad. Sci. USA 86*. 6597–6601.
Zasloff. M. 11992) Antibiotic peptides as mediators of innate immunity. *Curr. Opiri. Immunol. 4*. 3–7.
Zasloff. M., Martin. B. & Chen. H.-C. (1988) Antimicrobial activity of synthetic magainin peptides and several analogues. *Proc. Natl Acad. Sci. USA 85*. 910–913.