Available online at www.sciencedirect.com
ScienceDirect
Colloids and Surfaces B: Biointerfaces 60 (2007) 236–242
COLLOIDS AND SURFACES B
www.elsevier.com/locate/colsurfb

Binding orientation and activity determinants of the antimicrobial peptide cryptdin-4 revealed by potency of mutants

Jason E. Cummings, T. Kyle Vanderlick *

Department of Chemical Engineering, Princeton University, Princeton, NJ 08544, Unites States

Received 5 June 2007; accepted 20 June 2007
Available online 29 June 2007

Abstract

Cryptdin-4 is a β-sheet antimicrobial peptide of the defensin family that is found in the immune system of mice. Several structure–activity studies of this peptide have previously been conducted, but none have been based on residue–membrane interactions as part of an overall hypothesis on the peptide's orientation in the membrane. We pursue this valuable approach by first using previously reported NMR structural data to propose a membrane-bound orientation of the peptide. Four mutants are then strategically designed to modulate membrane perturbative activity in a manner consistent with the proposed binding orientation. Membrane perturbation is evaluated using a simple fluorescence-based vesicle leakage assay using POPG to form the model membrane. Effects of peptide mutations are found to be consistent with the suggested binding orientation. This approach is successfully used to create synthetic peptides with enhanced or diminished ability to perturb membranes and also yields insights on the nature of peptide–membrane interactions.

Keywords: Peptide; Mutant; Leakage; Lipid; Membrane

1. Introduction

Antimicrobial peptides (AMPs) exist as part of the immune system of nearly every living thing, displaying cidal activity towards microbes that requires neither high specificity nor memory. This cidal action is thought to result from a generic attack on the lipid moieties of the pathogenic membrane. In 1989, Lehrer et al. were the first to propose a link between the bactericidal activity of AMPs and the membrane destabilization events resulting from their interactions with the surface of bacterial cells [1]. Subsequent studies by Wade et al. showed that the L and D enantiomers of many AMPs display similar biological activity, suggesting that the cidal mechanism of the peptides does not require any stereospecific interaction with receptors on the pathogenic membrane [2]. As a result, it is now widely accepted that the microbicidal activity of AMPs most likely occurs through non-specific, lethal interactions of the peptides with the lipid matrix of the pathogenic membrane.

Many AMPs share three traits that facilitate their ability to interact with and perturb pathogenic, generally anionic, membranes: (1) the presence of positively charged residues; (2) a large proportion of hydrophobic residues, and (3) the ability to fold into tertiary structures that segregate these hydrophilic (i.e., charged) and hydrophobic residues into distinct patches on the peptide's molecular surface. These characteristics control AMP activity and allow the peptides to optimally partition themselves into or onto membranes in a manner that disrupts membrane order and increases membrane permeability. Structure–activity studies that alter one or more of these shared traits (via amino acid residue mutations) have demonstrated the impact that such changes can have on the extent of AMP interactions with lipid bilayers [3–32].

Often these structure–activity studies have revealed that amino acid mutations are particularly effective when they are directed at specific residue positions, such as the N- or C- termini [3,4,7,11,15–17,19–21,24,26,28]. These findings suggest that, for a given peptide, certain residue positions are the critical determinants of its membrane disruptive activity and microbicidal potency. Some researchers even make the generalization that entire classes of peptides share the same "activity sites". Unfortunately, this peptide-centric line of thought has limited validity

* Corresponding author. Tel.: +1 609 258 4891; fax: +1 609 258 0211.
E-mail address: vandertk@princeton.edu (T.K. Vanderlick).

0927-7765/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.colsurfb.2007.06.023

and one needs to consider structural mutations in the context of the peptide's interactions with the membrane.

The notion of taking the importance of the membrane into consideration when designing peptide mutants has had success with peptides assuming an α-helix [5,6,8,10,12,30–32]. This is partly because this simple structure often clearly segregates the hydrophobic and hydrophilic residues of the peptide, thereby making it apparent how α-helical peptides will orient themselves upon binding to a lipid membrane. For β-sheet peptides however, the separation of hydrophilic and hydrophobic residues may not be as distinct as in α-helical peptides. Consequently this idea of accounting for the role of the membrane in mutant design has not been extended to peptides assuming a β-sheet structure.

Particularly important within the class of β-sheet peptides is a family of AMPs known as defensins. These ubiquitous peptides have been studied extensively in various peptide–membrane interaction experiments [33–39]. Here we are interested in a defensin found in mice known as cryptdin-4 (Crp4). This peptide possesses a net charge of +8.5 and has displayed the greatest potency of all mouse defensins that have been identified and tested for biological activity [40]. The research group of Andre Ouellette at UC-Irvine and their collaborators have previously shown that charge reversal mutations to Crp4 greatly attenuate its interaction with model membranes as well as its bactericidal activity [25]. Other experiments where they mutated the N-terminal residue of Crp4 or eliminated the secondary structure formation of the peptide showed that these changes altered the peptide's membrane perturbative ability only slightly and still allowed these mutants to exhibit bactericidal activity [13,23]. All of these structure–activity studies were based on making mutations to suspected activity sites.

In this paper, we create Crp4 peptide mutants not through the alteration of suspected activity sites, but based on the idea that membrane perturbation is a non-specific process where mutations to sites that are most intimate with the membrane will have the most profound and insightful effect on peptide activity. This approach, of course, relies upon a proposed orientation of interaction of the peptide with the membrane and allows us to test that hypothesis. If the proposed orientation is correct, a limited number of mindful mutations to sites that are expected to be in proximity to the membrane should yield a noticeable and predictable effect on peptide activity. We characterize the behavior of Crp4 peptide mutants using model membranes in the form of lipid vesicles. A fluorescence-based vesicle leakage assay reveals the significance of the mutations and the likelihood that the proposed binding orientation is correct.

2. Materials and methods

2.1. Peptide

All peptides were purchased from New England Peptide, Inc. (Gardner, MA). The peptides were synthesized by solid phase peptide synthesis and were verified by HPLC and amino acid analysis. All were >95% pure.

2.2. Lipids

1-Palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-rac-(1-glycerol)] (POPG) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) were all purchased from Avanti Polar Lipids (Alabaster, AL).

2.3. Chemicals and reagents

8-Aminonaphthalene-1,3,6-trisulfonic acid (ANTS) and *p*-xylene-bis-pyridinium bromide (DPX) were purchased from Molecular Probes (Eugene, OR). Unless otherwise stated, all other chemicals were from Sigma–Aldrich (St. Louis, MO), of the highest grade available, and used as received. Water was produced by a Milli-Q UF unit (Millipore, Bedford, MA) and had a resistivity of 18.2 MΩ cm.

2.4. Preparation of lipid vesicles

Large unilamellar vesicles (LUVs) were prepared by hydrating a dried lipid film with 3 mL of buffer. The lipid suspension was vortexed, frozen, and thawed for five cycles. The suspension was then extruded (Lipex Biomembranes Inc.) two times through a 400 nm (diameter) polycarbonate filter (Nucleopore Co.), followed by 10 times through a 100 nm (diameter) polycarbonate filter, which generates vesicles of the nearly that same size. Lipid concentrations were determined using a phosphorus assay explained in a procedure made available by Avanti Polar Lipids (www.avantilipids.com).

2.5. Fluorescence-based vesicle leakage assays

All peptides were tested for their relative abilities to induce leakage from large unilamellar phospholipid vesicles (LUVs) of defined composition. POPG LUVs and POPC LUVs were loaded with a fluorophore/quencher (ANTS/DPX) system at quenched conditions [41]. Dried lipid films were hydrated with aqueous solutions consisting of 17 mM ANTS, 60.5 mM DPX, 10 mM HEPES, 31 mM NaCl, and 19.5 mM NaOH (260 mOsm/L, pH 7.4) and subjected to the procedure above (see Section 2.4). Vesicles were separated from unencapsulated ANTS/DPX by gel-permeation chromatography with 130 mM NaCl, 10 mM HEPES, and 4.5 mM NaOH (260 mOsm/L, pH 7.4) as column eluant. Vesicular suspensions diluted with eluant buffer to 75 μM of total lipid were incubated with peptide at ambient temperature up to a peptide to lipid molar ratio of 0.05. Fluorescence produced by ANTS release was monitored at 520 nm (excitation at 353 nm). Fluorescence values after 4 h were expressed relative to fluorescence obtained by vesicular solubilization with Triton X-100.

3. Results and discussion

The creation of Crp4 mutants for this study was based on the idea that peptide-induced membrane perturbation is a generic process where the amino acid residues in close contact with the membrane are the agents most responsible for the membrane-

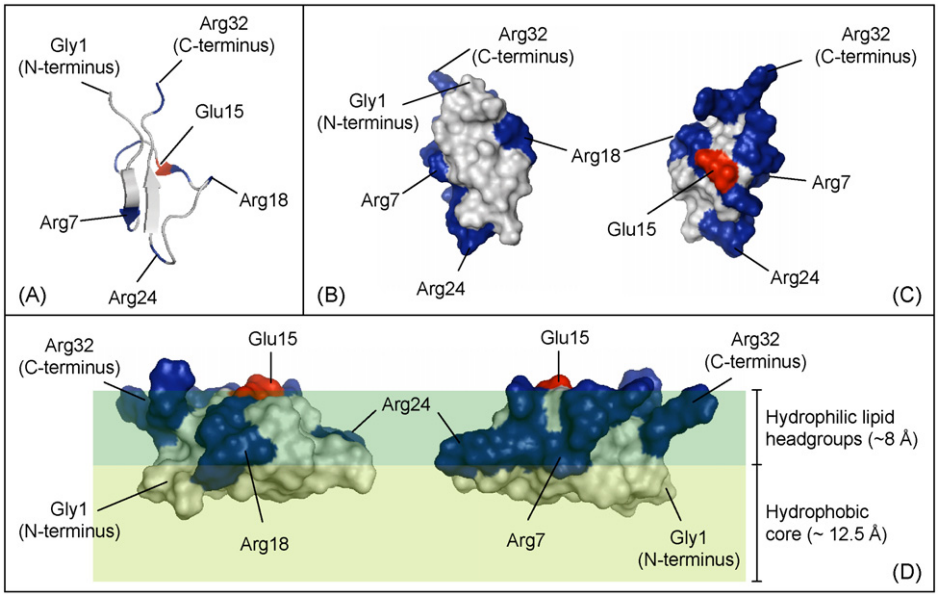

Fig. 1. (A) Ribbon diagram of Crp4. (B) Surface structure of the hydrophobic face of Crp4 (same perspective as panel A). (C) Surface structure of the hydrophilic face of Crp4. (D) Two different perspectives (180° rotation about the *y*-axis) of the proposed binding orientation of Crp4 upon adsorption to a lipid membrane. A lipid monolayer is represented by the light and dark green regions surrounding the peptide molecule. In each panel, residues with cationic side chains are shown in blue while those with anionic side chains are shown in red. White residues contain uncharged side chains. Each of these structures was produced with PyMOL [43].

disruptive activity of the peptide; perturbation is not the result of intrinsic activity sites, such as the N- or C-termini. To support this idea we first propose an orientation of Crp4 with the membrane upon binding. Crp4 mutants are then designed with mindful changes to the peptide in specific residue positions suspected to be in close proximity to the membrane. The activity of these mutants is evaluated with fluorescence-based vesicle leakage assays. Results from these assays ultimately allow us to confirm or refute our proposed orientation of Crp4 upon binding to the membrane.

NMR experiments with Crp4 have previously revealed the folded β-sheet structure of the peptide (Fig. 1A) and manifested the spatial arrangement of hydrophobic and hydrophilic residues [42]; to a large extent, one face of the peptide is hydrophobic (Fig. 1B) while the other is hydrophilic (Fig. 1C). We have used this information to hypothesize the most likely membrane-bound orientation of Crp4 as shown in Fig. 1D. The proposed orientation places the apolar face of the peptide surface into the hydrophobic core of the membrane bilayer. The positively charged residues of Crp4 lie amongst the hydrophilic lipid headgroups and the aqueous surroundings, while the lone anionic residue of Crp4 is positioned where it is most isolated from the membrane. With this model as our basis, we have made mutations to Crp4 that are expected to either favorably or adversely impact its ability to interact with a lipid membrane. At the same time, this procedure provides a way to confirm the peptide's proposed orientation.

Four mutants were designed for this study with altered charge and/or hydrophobicity. These are summarized in Table 1 and shown in Figs. 2A, 3A, 4A, and 5A. One mutant was designed to be significantly more perturbative resulting from an enlargement of the hydrophobic face of the peptide ((G1LL)-Crp4). A second mutant was designed to increase membrane perturba-

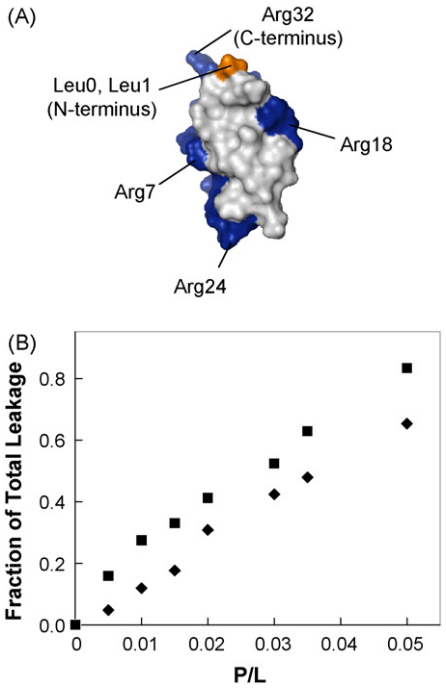

Fig. 2. (A) Surface structure of (G1LL)-Crp4 where the location of the added leucines (labeled as Leu0 and Leu1) is shown in orange. Residues with cationic side chains are shown in blue while residues with uncharged side chains are shown in white. This structure was produced with PyMOL [43]. (B) Addition of two leucines at the N-terminus of Crp4 in place of glycine increases the hydrophobicity and membrane perturbative activity of the peptide. The newly added hydrophobic residues in this position allow them to effectively penetrate into the POPG membrane core and induce greater leakage than the wildtype peptide. (♦) Crp4 and (■) (G1LL)-Crp4.

Table 1
The primary structure of Crp4 is listed, followed by the names of each designed mutant along with a description and physical effect of the mutation made

Peptide name		Crp4
Peptide sequence		GLLCY CRKGH CKRGE RVRGT CGIRF LYCCP RR
Mutant name	Mutation description	Physical effect of mutation
(G1LL)-Crp4	N-terminal glycine replaced by two leucines	Increases hydrophobicity
(K8,R24L)-Crp4	Lysine in eighth position and arginine in 24th position replaced by leucines	Reduces peptide charge to +6.5 but increases hydrophobicity
(Y27E)-Crp4	Tyrosine in 27th position replaced by glutamate	Reduces peptide charge to +7.5
(T20R)-Crp4	Threonine in 20th position replaced by arginine	Increases peptide charge to +9.5

tion via two charge-to-hydrophobic mutations which hopefully would allow individual peptide molecules to tilt themselves deeper into the membrane while still maintaining sufficient charge for nearly complete binding of peptide ((K8,R24L)-Crp4). The third mutant was designed to be significantly less perturbative as a result of substituting an anionic charge within the hydrophobic face ((Y27E)-Crp4). The final mutant was designed to demonstrate that increases in peptide charge do not necessarily lead to increased membrane perturbation ((T20R)-Crp4).

The membrane disruptive activities of Crp4 and all mutants were assessed by examining peptide-induced leakage of low molecular weight fluorophores (i.e., ANTS) from LUV (see Section 2). Vesicle leakage was monitored by measuring changes in the fluorescence intensity of the sample as fluorophore escaped the interior of vesicles after addition of peptide. Although the results of vesicle leakage assays are often corroborated by the results of bactericidal assays (which in simplest terms identify the inhibitory and/or cidal concentration of perturbant), we did not conduct the latter because the complexities of biological membranes can sometimes obscure direct comparison of biological with biophysical assays. In addition, in comparison to the well controlled vesicle leakage assays, the bactericidal assays provide only an undifferentiated measure of cell perturbation (i.e., death or no death).

Before discussing the specific effects of our mutations on peptide perturbative activity, we must first address the degree

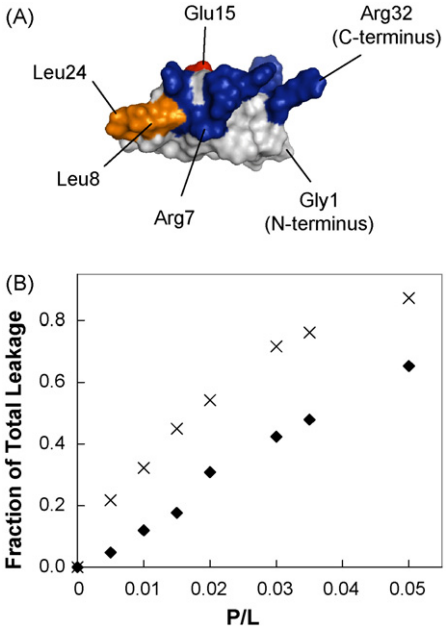

Fig. 3. (A) Surface structure of (K8,R24L)-Crp4 where the locations of the added leucines (labeled as Leu8 and Leu24) are shown in orange. Residues with cationic side chains are shown in blue while those with anionic side chains are shown in red. White residues contain uncharged side chains. This structure was produced with PyMOL [43]. (B) Replacing two cationic residues of Crp4 (i.e., lysine in the 8th position and arginine in the 24th position) with leucine residues reduces the overall charge of the peptide but increases its hydrophobicity. Although less charge is often associated with less binding, the replaced cationic residues are apparently unnecessary for complete binding of peptide; (K8,R24L)-Crp4 displays activity greater than the wildtype peptide and even greater than any other mutant designed for this study when interacting with POPG vesicles. The large increase in peptide activity suggests that residues 8 and 24 are positions of optimal interaction with the membrane. (♦) Crp4 and (×) (K8,R24L)-Crp4.

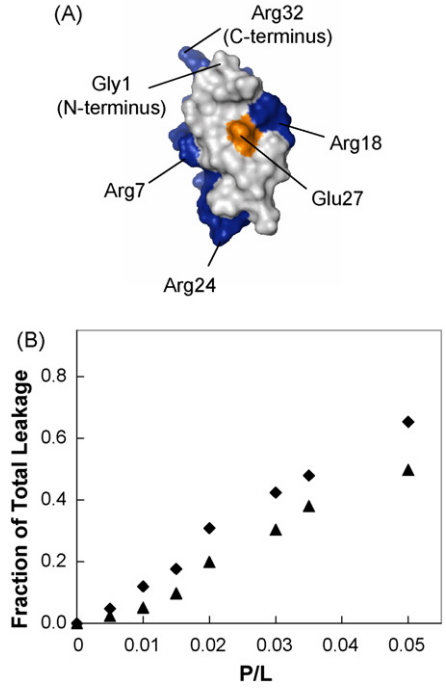

Fig. 4. (A) Surface structure of (Y27E)-Crp4 where the location of the added glutamate (labeled as Glu27) is shown in orange. Residues with cationic side chains are shown in blue while residues with uncharged side chains are shown in white. This structure was produced with PyMOL [43]. (B) The presence of the anionic residue glutamate is in a position that should reduce the insertion of the peptide belly into the hydrophobic membrane core. Indeed the reduced activity of (Y27E)-Crp4 with respect to wildtype Crp4 is a testament to this occurrence with POPG membranes. (♦) Crp4 and (▲) (Y27E)-Crp4.

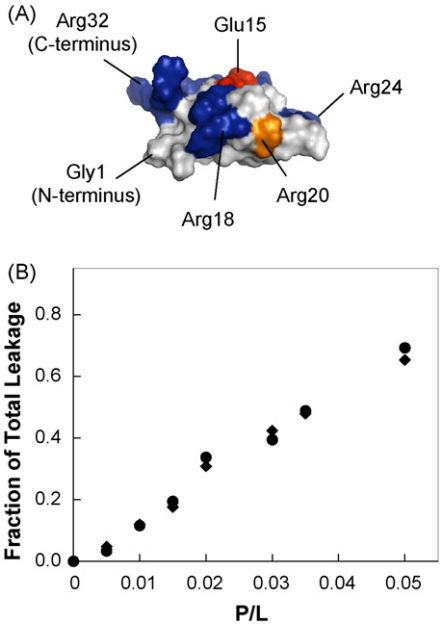

Fig. 5. (A) Surface structure of (T20R)-Crp4 where the location of the added arginine (labeled as Arg20) is shown in orange. Residues with cationic side chains are shown in blue while those with anionic side chains are shown in red. White residues contain uncharged side chains. This structure was produced with PyMOL [43]. (B) Because wildtype Crp4 already experiences complete binding at the P/L ratios tested, the heightened charge of (T20R)-Crp4 does not increase the quantity of bound peptide. Furthermore, the position of the newly added arginine does not noticeably alter the depth of peptide insertion into the membrane. Consequently (T20R)-Crp4 displays identical activity as wildtype Crp4 when interacting with POPG vesicles. (♦) Crp4 and (●) (T20R)-Crp4.

of peptide binding and possible differences in such between mutants. The molar ratios of peptide to lipid were maintained between 0 and 0.05, which are conditions where the wildtype peptide exhibits >90% binding to highly anionic POPG vesicles (as determined using a tryptophan fluorescence binding assay [44]). Because the mutants we have chosen for this study are not very different from the wildtype peptide, we are confident that nearly complete binding also holds for each of these peptides, which all still have a very high net positive charge (i.e., >6). For example, as will be shown, even a ∼25% decrease in peptide charge can still lead to an increase in peptide perturbative activity. Therefore, the results of our vesicle leakage assays can be viewed as a direct indicator of the change in perturbative activity due to variations in the net charge or hydrophobicity of the peptide and not an artifact of differences in binding. Results are thus reported in terms of total peptide to lipid ratios.

The first mutant evaluated in this study (G1LL)-Crp4, demonstrates the effect of increased hydrophobicity on the extent of AMP interaction with anionic membranes. The hydrophobic side chains of the newly added leucine residues enlarge the size of the apolar face of the peptide (which resides within the hydrophobic membrane core) and should penetrate deeply into the bilayer (Fig. 2A). As a result, this mutant should more effectively disorder the lipid packing of the membrane and elicit a greater efflux of encapsulated POPG vesicle contents than the wildtype peptide. Indeed the results of vesicle leakage assays, as shown in Fig. 2B, demonstrate this occurrence. Clearly the strategic addition of leucines in a position where they can readily access the membrane core leads to such an increase in activity.

The second mutant designed for this work is a hybrid mutant containing both hydrophobic and electrostatic mutations. (K8,R24L)-Crp4, as shown in Fig. 3A, has a lowered cationic charge of +6.5 but greater net hydrophobicity due to the substitutions of a lysine residue (cationic) and an arginine residue (cationic) with two leucines (hydrophobic). Addition of the two leucines not only introduces two apolar side chains that can penetrate into the membrane core, but likely will also allow the peptide as a whole to partition deeper into the bilayer due to the removal of two highly polar side chains. As a result, we expect the activity of this peptide to increase, assuming that the reduced charge of this mutant is still sufficient to allow nearly complete peptide binding. As can be seen in Fig. 3B (K8,R24L)-Crp4 displays activity greater than the wildtype peptide and even greater than any of the mutants designed for this study when interacting with POPG vesicles, despite a reduction in charge by nearly 25%. The heightened activity of this mutant above all the other mutants suggests that the replaced cationic residues are unnecessary for nearly complete binding of peptide to the membrane at the P/L ratios tested.

(Y27E)-Crp4 is a mutant that demonstrates the critical importance of the positions of polar/charged residues in the peptide structure in the context of the protein's ability to adopt a structure segregating hydrophobic and hydrophilic residues. The anionic glutamate of (Y27E)-Crp4 is near the center of the hydrophobic belly of the peptide (Fig. 4A). According to our proposed membrane-bound orientation of Crp4, this mutation is in a position that would reduce the insertion of the peptide belly into the hydrophobic membrane core and subsequently its membrane perturbative activity. As hypothesized, Fig. 4B shows that the activity of (Y27E)-Crp4 is in fact weakened with respect to the wildtype peptide. It could be argued that this attenuation of activity is simply due to a lower net peptide charge. However, when one realizes that the least charged mutant of this study (K8,R24L)-Crp4, is nearly twice as potent as (Y27E)-Crp4, the commonly observed correlation between net peptide charge and membrane perturbation is not a sufficient explanation for describing the behavior of (Y27E)-Crp4.

The final peptide used for this study (T20R)-Crp4 is the only mutant with a charge greater than that of the wildtype peptide; in this case, the slightly polar threonine is replaced with a cationic arginine (Fig. 5A). Despite the increase in peptide charge, this mutation is not expected to increase membrane perturbation for two key reasons. First, the binding is already complete in the less-charged native state. Second, the mutation is strategically placed near the hydrophilic lipid headgroups so as not to affect the membrane-bound orientation of the peptide. If anything, one might imagine that the placement of this cationic charge would resist penetration into the membrane core and tilt the peptide out of the bilayer. This mutation produces no apparent change in peptide activity, more or less as expected (Fig. 5B).

Leakage assays were also conducted with each mutant using vesicles constructed entirely of the zwitterionic lipid POPC.

Like Crp4, none of the mutants showed any activity whatsoever against these vesicles at the same concentrations over which substantial leakage occurred with POPG vesicles (data not shown). The electrostatic attraction between Crp4 and the POPC lipid membrane is apparently not sufficient enough to facilitate peptide binding to the bilayer.

In addition to the mutants we have designed and tested for this study, there have also been a number of Crp4 mutants previously created and tested for activity by Ouellette and coworkers. We can now offer insight on the behavior of these mutants using our proposed membrane-bound orientation of Crp4. Two N-terminal mutants (des-Gly)-Crp4 and (G1R)-Crp4 used in both leakage assays and bactericidal assays showed only modest changes in activity [23]. Neither of these mutations would be expected to change the orientation or depth of insertion of the peptide. Indeed, the more highly cationic (G1R)-Crp4 might be expected to behave similar to (T20R)-Crp4 which shows no increased perturbation.

Other Crp4 mutants created by the Ouellette lab replaced multiple (i.e., 2 to 3) cationic residues of the peptide with anionic residues. These charge-reversal mutants, (R31D/R32D)-Crp4, (G1D/R31D/R32D)-Crp4, and (R16D/R24D)-Crp4, showed complete elimination of activity in both leakage assays and bactericidal assays despite still exhibiting a substantial net positive charge [25]. According to our proposed binding orientation, the charged residues of Crp4 lie within the charged lipid headgroup region of anionic vesicle membranes and anionic bacterial membranes. By replacing the cationic residues with anionic residues, not only is positive peptide charge attenuated but there are now strong repulsive forces between the membrane and regions of the peptide that are very near the anionic lipid headgroups. Therefore, the absence of activity displayed by the aforementioned charge reversal mutants is not surprising; complete binding may no longer prevail and those peptides that do bind may not be able to penetrate as deeply.

A third set of Crp4 mutants designed by the Ouellette research group examined the effect of attenuating the formation of peptide structure [13]. This was achieved by replacing some or all of the disulfide bridge-forming cysteine residues within the peptide sequence with alanine. Despite the absence of structure formation, these mutants were at least as active as wildtype Crp4 in both leakage assays and bactericidal assays. This is likely due to enhanced flexibility of the structureless mutants thereby allowing maximal interaction of the hydrophobic residues with the membrane.

The collective results of the assays conducted on the four mutants designed for this work and the results of assays conducted with previously designed Crp4 mutants provide evidence supporting our proposed orientation of binding of Crp4 to lipid membranes. The results also show that the activity of Crp4 is not based on specific activity sites nor on overall peptide characteristics such as net charge. Instead one must consider the placement of charge and hydrophobic residues in relation to the orientation of the peptide on the membrane. Only through such considerations can one expect to successfully design Crp4 mutants with optimal antimicrobial activity and minimal cytotoxicity.

4. Conclusion

The importance of charge, hydrophobicity, and structure formation to the activity of antimicrobial peptides is well recognized and has long been the focus of structure–activity studies of AMPs. These effects are important but only in the context of how they change the interactions of the peptide with the hosting membrane. So, for example, it is naive to assume, as is typically done, that blindly increasing or decreasing the net charge of an AMP might measurably affect its activity. As we show here, net charge can be increased without any impact on peptide-induced membrane perturbation while other strategic mutations that decrease peptide charge can lead to greater membrane perturbation. Small changes in peptide structure can have a significant impact on activity if they lead to changes in peptide binding and/or the orientation of bound peptide in the membrane. While the extent of binding is clearly associated with extent of membrane damage, this work shows that changes in orientation alone can also lead to measurable changes in activity. Studies of peptide mutations, in the framework used herein, cannot only lead to more effective microbicides but provides a platform for revealing the mechanisms of peptide activity.

Acknowledgement

This work was supported by a fellowship from the National Science Foundation to J.E.C.

References

[1] R.I. Lehrer, A. Barton, K.A. Daher, S.S. Harwig, T. Ganz, M.E. Selsted, J. Clin. Invest. 84 (1989) 553.
[2] D. Wade, A. Boman, B. Wahlin, C.M. Drain, D. Andreu, H.G. Boman, R.B. Merrifield, Proc. Natl. Acad. Sci. U.S.A. 87 (1990) 4761.
[3] E. Bikshapathy, N. Sitaram, R. Nagaraj, J. Pept. Res. 53 (1999) 47.
[4] S.E. Blondelle, R.A. Houghten, Biochemistry 30 (1991) 4671.
[5] S.E. Blondelle, R.A. Houghten, Biochemistry 31 (1992) 12688.
[6] I. Cornut, K. Buttner, J.L. Dasseux, J. Dufourcq, FEBS Lett. 349 (1994) 29.
[7] J.H. Cuervo, B. Rodriguez, R.A. Houghten, Pept. Res. 1 (1988) 81.
[8] M. Dathe, M. Schumann, T. Wieprecht, A. Winkler, M. Beyermann, E. Krause, K. Matsuzaki, O. Murase, M. Bienert, Biochemistry 35 (1996) 12612.
[9] M. Dathe, T. Wieprecht, Biochim. Biophys. Acta 1462 (1999) 71.
[10] M. Dathe, T. Wieprecht, H. Nikolenko, L. Handel, W.L. Maloy, D.L. MacDonald, M. Beyermann, M. Bienert, FEBS Lett. 403 (1997) 208.
[11] C.E. Dempsey, Biochim. Biophys. Acta 1031 (1990) 143.
[12] T. Kiyota, S. Lee, G. Sugihara, Biochemistry 35 (1996) 13196.
[13] A. Maemoto, X. Qu, K. Rosengren, H. Tanabe, A. Henschen-Edman, D.J. Craik, A.J. Ouellette, J. Biol. Chem. 279 (2004) 44188.
[14] W.L. Maloy, U.P. Kari, Biopolymers 37 (1995) 105.
[15] R.B. Merrifield, E.L. Merrifield, P. Juvvadi, D. Andreu, H.G. Boman, Ciba Found. Symp. 186 (1994) 5.
[16] A. Mor, P. Nicolas, J. Biol. Chem. 269 (1994) 1934.
[17] H. Mozsolits, H.J. Wirth, J. Werkmeister, M.I. Aguilar, Biochim. Biophys. Acta 1512 (2001) 64.
[18] A.J. Ouellette, D.P. Satchell, M.M. Hsieh, S.J. Hagen, M.E. Selsted, J. Biol. Chem. 275 (2000) 33969.
[19] K.L. Piers, M.H. Brown, R.E. Hancock, Antimicrob. Agents Chemother. 38 (1994) 2311.
[20] P.A. Raj, K.J. Antonyraj, T. Karunakaran, Biochem. J. 347 (2000) 633.

[21] W.E. Robinson Jr., B. McDougall, D. Tran, M.E. Selsted, J. Leukoc. Biol. 63 (1998) 94.
[22] D. Satchell, T. Sheynis, Y. Shirafuji, S. Kolusheva, A. Ouellette, R. Jelinek, J. Biol. Chem. 278 (2003) 13838.
[23] D.P. Satchell, T. Sheynis, S. Kolusheva, J. Cummings, T.K. Vanderlick, R. Jelinek, M.E. Selsted, A.J. Ouellette, Peptides 24 (2003) 1795.
[24] Y. Shai, Toxicology 87 (1994) 109.
[25] H. Tanabe, X. Qu, C.S. Weeks, J.E. Cummings, S. Kolusheva, K.B. Walsh, R. Jelinek, T.K. Vanderlick, M.E. Selsted, A.J. Ouellette, J. Biol. Chem. 279 (2004) 11976.
[26] S. Thennarasu, R. Nagaraj, Protein Eng. 9 (1996) 1219.
[27] A. Tossi, L. Sandri, A. Giangaspero, Biopolymers 55 (2000) 4.
[28] M. van Veen, G.N. Georgiou, A.F. Drake, R.J. Cherry, Biochem. J. 305 (1995) 785.
[29] S.H. White, W.C. Wimley, Biochim. Biophys. Acta 1376 (1998) 339.
[30] T. Wieprecht, M. Dathe, M. Beyermann, E. Krause, W.L. Maloy, D.L. MacDonald, M. Bienert, Biochemistry 36 (1997) 6124.
[31] T. Wieprecht, M. Dathe, R.M. Epand, M. Beyermann, E. Krause, W.L. Maloy, D.L. MacDonald, M. Bienert, Biochemistry 36 (1997) 12869.
[32] T. Wieprecht, M. Dathe, E. Krause, M. Beyermann, W.L. Maloy, D.L. MacDonald, M. Bienert, FEBS Lett. 417 (1997) 135.
[33] I. Cipakova, E. Hostinova, Biologia 58 (2003) 335.
[34] A.I. Hobta, P.V. Pogrebnoy, Exp. Oncol. 20 (1998) 170.
[35] D.M. Hoover, Z.B. Wu, K. Tucker, W.Y. Lu, J. Lubkowski, Antimicrob. Agents Chemother. 47 (2003) 2804.
[36] K. Hristova, M.E. Selsted, S.H. White, Biochemistry 35 (1996) 11888.
[37] K. Hristova, M.E. Selsted, S.H. White, J. Biol. Chem. 272 (1997) 24224.
[38] M. Mandal, R. Nagaraj, J. Pept. Res. 59 (2002) 95.
[39] P.A. Raj, A.R. Dentino, FEMS Microbiol. Lett. 206 (2002) 9.
[40] A.J. Ouellette, M.M. Hsieh, M.T. Nosek, D.F. Cano-Gauci, K.M. Huttner, R.N. Buick, M.E. Selsted, Infect. Immunol. 62 (1994) 5040.
[41] M. Smolarsky, D. Teitelbaum, M. Sela, C. Gitler, J. Immunol. Methods 15 (1977) 255.
[42] W. Jing, H.N. Hunter, H. Tanabe, A.J. Ouellette, H.J. Vogel, Biochemistry 43 (2004) 15759.
[43] W.L. Delano, The Pymol User's Manual, DeLano Scientific, Palo Alto, CA, USA, 2002.
[44] A.S. Ladokhin, S. Jayasinghe, S.H. White, Anal. Biochem. 285 (2000) 253.