Biochimica et Biophysica Acta 1828 (2013) 2479–2486

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

# Flexibility is a mechanical determinant of antimicrobial activity for amphipathic cationic α-helical antimicrobial peptides

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ARTICLE INFO

*Article history:*
Received 3 November 2012
Received in revised form 2 June 2013
Accepted 13 June 2013
Available online 24 June 2013

*Keywords:*
AMP
Flexibility
B-factor
Antimicrobial activity
Structure–activity relationship

ABSTRACT

Antimicrobial peptides (AMPs) are recognized as the potential substitutions for common antibiotics. Flexibility has been demonstrated to be a dominant on antimicrobial activity of an AMP, similar to the structural parameters such as hydrophobicity and hydrophobic moment as well as positive charge. To better understand the effect of flexibility on antimicrobial activity, we herein examined seventy-eight peptides derived from nine different species. Defined as a weighted average of amino acid flexibility indices over whole residue chain of AMP, flexibility index was used to scale the peptide flexibility and indicated to be a reflection of mechanical properties such as tensile and flexural rigidities. The results demonstrated that flexibility index is relevant to but different from other structural properties, may enhance activity against *Escherichia coli* for stiff clustered peptides or reduce activity against *E. coli* for flexible clustered peptides, and its optimum occurs at about −0.5. This effect of flexibility on antimicrobial activity may be involved to the antimicrobial actions, such as stable peptide-bound leaflet formation and sequent stress concentration in target cell membrane, mechanically. The present results provide a new insight in understanding antimicrobial actions and may be useful in seeking for a new structure–activity relationship for cationic and amphipathic α-helical peptides.



## 1. Introduction

Antimicrobial peptides (AMPs), "the ancient players in innate immunity" [1–3], are the effective defensive weapons especially in insects, invertebrates and other species [4]. It is believed that, AMPs are one of the first defensive lines against a pathogenic skin fungus, *Batrachochytrium dendrobatidis*, which is involved in global amphibian declines [5,6]; and through inducing selective innate immune responses with diverse host cellular processes such as cytokine release, chemotaxis, antigen presentation, etc., AMPs function as a selective controller of inflammation, a protector against infection and an enhancer of wound healing as well as an initiator of adaptive immune responses [7–9]. For example, the human cationic peptide LL-37 modifies dendritic cell differentiation and bridges both innate- and adaptive-immune responses [10]; and, the β-defenses recruit both dendritic and T cells to the microbial invasion site through interaction with CCR6 [11].

Much attention has been paid on AMPs, because of not only their broad activities against various pathogens but also their medicinal potential as substitutions of common antibiotics [8]. A peptide generally has amino acid chain length of dozens, and its antimicrobial activity is relevant to structural parameters, such as conformation, charge, hydrophobicity, amphipathicity and polar angle [12,13]. A well-understood antimicrobial mechanism is referred to non-receptor-mediated interaction of AMPs and target cell membrane [8]. The peptides reach cell membrane under electrostatic driving force first [14] and then interact with membrane by their hydrophobic faces [15], possibly undergoing a conformational change [12]; subsequently, the peptides disrupt or insert into membrane further when the peptide-to-lipid ratio is above a threshold [16]; finally, the cell is killed due to the lysing of membrane or disturbance of cellular components [1,7,12].

Flexibility is another structural determinant of antimicrobial activity. An AMP may endeavor to attain a compromise between flexibility and stability for its function realization, like proteins [17,18]. A hinge near the central position of an α-helical chain may be beneficial for the peptide to span the lipid bilayer, and play important roles in bacterial cell selectivity, and antimicrobial and antitumor activities [19–22]. Recently, we demonstrated that the antimicrobial activity towards *Candida albicans*, *Staphylococcus aureus* or *Escherichia coli* may increase with Young's modulus (*EI*) for peptide HP(2–20) and its analogs [23,24].

But so far, less knowledge lies not only in flexibility effects on antimicrobial actions but also in flexibility itself for various AMPs

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0005-2736/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bbamem.2013.06.017

derived from different species, because of lack of crystal structure data of these peptides. It is believed that, B-factor or temperature factor, which is linearly related to the mean square displacement of an atom in protein crystal structure, can be used to rate the protein flexibility, thermal stability and intrinsic disorder [25,26]. With normalized B-factors of $C^{\alpha}$-atoms from thirty-one refined protein structures, Karplus and Schulz presented a relationship of B-factor and amino acid type by a nearest-neighbor analysis [27]; Vihinen et al. showed no distinct differences in B-factors of $C^{\alpha}$- and backbone-atoms within ninety-two structures [28]; and by examining 290 protein chains, Smith et al. found B-factor obeying the Gumbel distribution rather than the normal distribution [29]. These well-characterized B-factors of amino acid residues should be useful in predicting the flexibilities of the AMPs with or without crystal structure data. Besides, the elastic modulus of the amphipathic α-helical peptides can be estimated through steered molecular dynamic simulation [24]. Physically, the peptide modulus might be related to the flexibility profile of residues in the peptide. However, the structure–activity relationship is not monolithically parametric due to the interdependent structural parameters [12,30], leading to another barrier in uncovering flexibility effects on antimicrobial actions.

To understand whether and how flexibility governs antimicrobial activities of AMPs derived from different species, we herein examined seventy-eight peptides clustered into nine different groups, namely the group Maculatin 1.1 [31], Brevinin-1 [32], pseudin-2 [33], ranatuerin-1 [34], HP(2–20) [23], CAP18 [35], Tritrp1 [30] and MIIa [36] as well as the group IsCT [37]. Each group consists of one native peptide and its analogs with the same chain length (Materials and methods; Table A in Appendices). With the published data of the amino acid flexibility indices [29], the overall flexibility of each peptide was evaluated through a weighted average of the amino acid flexibility indices over the whole residue chain, and related to its other structural properties and the antimicrobial activity against *E. coli*. The present results showed that flexibility reflects two mechanical properties such as tensile modulus and flexural resistance for the cationic amphipathic α-helical peptides, and is a dominant determinant of antimicrobial activity in comparison with its partners such as charge and hydrophobicity as well as hydrophobic moment. For antimicrobial function realization, flexibility serves as either an activity enhancer for the rigid group Maculatin 1.1, Brevinin-1 and pseudin-2 or an activity reducer for the flexible group ranatuerin-1, HP(2–20) and CAP18. This may be involved in the stable peptide-bound leaflet formation and the sequent stress concentration in target cell membrane.

## 2. Materials and methods

### 2.1. AMPs and their minimum inhibition concentrations

We used the minimum inhibition concentration (MIC) aimed at *E. coli* to assess the antimicrobial activities of AMPs. The data of MICs and sequences of seventy-eight peptides in nine AMP groups, namely the peptide group Maculatin 1.1 [31], Brevinin-1 [32], pseudin-2 [33], ranatuerin-1 [34], HP(2–20) [23], CAP18 [35], Tritrp1 [30], MIIa [36] and IsCT [37] (Table A in Appendices), were taken from the published works, in which the antimicrobial assays of these peptides were described in detail. These peptide groups, respectively, have 5, 8, 15, 17, 7, 6, 8, 6 and 6 members of same chain length. Differences among the members in each cluster lie just in one or more amino acid residues being replaced (Table A in Appendices). Maculatin 1.1 was from the skin glands of the tree frog, *Litoria genimaculata* [31]; Brevinin-1Bb and its analogs were purified from *Rana berlandieri* and *Rana pipiens* [32]; the peptides in group Pseudin-2 were isolated from the skin of the South American paradoxical frog, *Pseudis paradoxa* [33]; Ranatuerin-1 and its analogs were derived from the skin of the bullfrog, *Rana catesbeiana* [34]; HP(2–20) came from *Helicobacter pylori* [23]; CAP18 was identified from rabbit granulocytes [35]; Tritrp1 was found in porcine cathelicidin cDNA [30]; MIIa was from Xenopus skin [36]; and IsCT was isolated from the scorpion, *Opisthacanthus madagascariensis* [37]. All the peptides were cationic and amphipathic. $MIC^{-1}$ was defined as the antimicrobial activity index to reflect the antimicrobial activities of these peptides directly, for simplicity.

### 2.2. Sequence alignment, structural parametric estimation and conformational analysis

The hydrophobicity ($H$) and hydrophobic moment ($\mu H$) were calculated by HELIQUEST (http://heliquest.ipmc.cnrs.fr/) [38]. The charge density ($\rho$) of a peptide was defined as the ratio of the positive charge number to the chain length of the peptide. Sequence alignment was performed with Clustal W2 (http://www.ebi.ac.uk/Tools/clustalw2/) [39]. Maculatin 1.1, Brevinin-1Bb, Pseudin-2, ranatuerin-1, HP(2–20), CAP18, Tritrp1, MIIa and IsCT were assigned as the native peptides in the nine groups, respectively. The alignment scores were read from data of pairwise sequence alignment of a native peptide to its analogs. The data of crystal structures of some peptides, such as HP(2–20), Tritrp1, IsCT, CAP18 and MIIa as well as a part of analogs of HP(2–20), Tritrp1 and IsCT, were downloaded from RCSB Protein Data Bank (http://www.pdb.org/pdb/home/home.do). The PDB codes were 1P0J (HPA1), 1P0G (HP(2–20)), 1P0L (HPA2), 1P0O (HPA3), 1P5L (HPA5), 1LYP (CAP18), 2I1D (Tritrp1), 2I1E (Tritrp2), 2I1F (Tritrp3), 2I1G (Tritrp5), 2I1H (Tritrp7), 2I1I (Tritrp8), 2MAG (MIIa), 1T51 (IsCT), 1T52 ([$K^7$]-IsCT), 1T54 ([$A^6$]-IsCT) and 1T55 ([$K^7$, $P^8$, $K^{11}$]-IsCT). CAP18 was a fragment of the structure recorded in 1LYP. The visual molecular dynamics (VMD) package was used for visualization and picking out the corresponding fragment [40].

### 2.3. Calculations of flexibility indices of AMPs

The amino acid flexibility index was estimated by the corresponding location parameter (λ) of B-factor's Gumbel distribution for each of 20 amino acids, and were well quantified through fitting the data of the B-factors of the amino acid in 290 protein chains to Gumbel distribution [29]. The flexibility of a residue in an amino acid chain is dependent on whether the neighbor(s) of the residue is rigid or flexible, because a rigid neighbor decreases the residue flexibility and a flexible one does the opposite [27–29].

We herein define the peptide flexibility index ($F$) as a weighted average of amino acid flexibility index profile over whole residue chain of the peptide. To consider the neighbor effects on the amino acid flexibility, a sliding hat-shaped window with length of $n$ (=1, 3 or 5) was herein used to evaluate the values of $F$ [28,29]. The best window length referred in the previous studies was not considered in this study, because of the short lengths of the residue chains for the nine AMP groups. By reading the means of fitting results of λ from the work of Smith et al. [29] and assigning these λ-values to each residues in a residue chain [27,28], the peptide flexibility index $F$ was calculated by the following equations [27,28]:

$$F1 = \frac{1}{L}\sum_{j=1}^{L}\lambda_j \tag{1}$$

$$F_3 = \frac{1}{2(L-2)}\sum_{j=2}^{L-1}\left[\lambda_j + \frac{1}{2}\left(\lambda_{j-1} + \lambda_{j+1}\right)\right] \tag{2}$$

$$F_5 = \frac{1}{3(L-4)}\sum_{j=3}^{L-2}\left[\lambda_j + \frac{2}{3}\left(\lambda_{j-1} + \lambda_{j+1}\right) + \frac{1}{3}\left(\lambda_{j-2} + \lambda_{j+2}\right)\right] \tag{3}$$

where $F_n$ expresses the peptide flexibility index $F$ estimated with a sliding hat-shaped window with length of $n$ (=1, 3 and 5), $L$ is the

residue number of the peptide, and $\lambda_j$ is the location parameter of fitted B-factor's Gumbel distribution for the $j$th residue in the residue chain with $j \le L$. To test whether $F$ was sensitive to the window length choice or not, Pearson correlation coefficients of $F_1$, $F_3$ and $F_5$ were calculated. Statistical analyses were performed with Student's $t$ test for different groups.

### 2.4. Estimation of Young's modulus E, bending rigidity EI and persistence length $\xi_p$

It was postulated that, each peptide is an "ideal" chain and can be modeled by a uniform rod with Young's modulus of $E$ and radius of $a$. A mean value of $a$ of 0.25 nm, which was measured from five linear cationic α-helical peptides in group HP(2–20), was assigned to each peptide, for simplicity. Then, the cross section inertia moment $I$ for each rod-like peptide was read as 0.0031 $nm^4$ with $I = \pi a^4/4$, and the bending rigidity $\kappa_f$, the products of $E$ and $I$, was calculated through a presumption that Young's modulus $E$ is linearly dependent on flexibility index $F$, that is

$$E = \alpha \times F + \beta \tag{4}$$

where, the coefficient $\alpha$ and $\beta$ are not species-dependent, and read, respectively, to be $-22.78$ and $-2.66$ ($10^9$ $N/m^2$) through fitting the data of $E$ and $F$ of peptides in group HP(2–20) (Fig. 2). The persistence length $\xi_p$ was evaluated by $\xi_p = \kappa_f/k_bT$, where $k_b$ is the Boltzmann constant and $T$ is the absolute room temperature (= 310 K) [41]. The mean contour length $L_c$ of 3.03 nm for five linear cationic α-helical peptides in group HP(2–20) with the same chain length of nineteen residues [27,28] shows that each residue in these peptides contributes about 0.16 nm to $L_c$. This length of 0.16 nm per one residue was used to approximately measure the contour length $L_c$ for each peptide.

## 3. Results

### 3.1. Flexibility index, its distribution and species-dependent features

We calculated flexibility index ($F$), hydrophobicity ($H$) and hydrophobic moment ($\mu H$) as well as positive charge density ($\rho$), and performed sequence alignments to examine sequence similarities between the native peptides and their respective diverse analogs, for each AMP group (Table A and B in Appendices). The values of $F$ were evaluated with the location parameters of the fitted Gumbel distributions of B-factors of 20 amino acids [27–29], in three weighted sliding windows with lengths of 1, 3 and 5, respectively [27,28] (Materials and methods). Pearson correlation coefficients of two peptide flexibility index sets evaluated in any two different sliding window lengths are larger than 0.978 (data not shown), showing less effect of sliding window length on calculation of $F$. So we herein chose the sliding window length of 3 to estimate the flexibility index $F$ as a trade-off between the short chain lengths and the exceptional chain terminal flexibilities of the peptides [27,28]. All results were listed in Table 1, where the data were presented as means plus standard deviations (stds).

We found that, as a scale of flexibility, the flexibility indices of all 78 peptides range widely from $-0.715$ to $-0.134$ (Table B in Appendices), and approximately obey a Gaussian distribution with mean ± std of $-0.46 \pm 0.12$ (Fig. 1). The flexibility index $F$ has mean ± std of $-0.66 \pm 0.04$, $-0.57 \pm 0.01$, $-0.51 \pm 0.02$, $-0.48 \pm 0.03$, $-0.36 \pm 0.05$, $-0.23 \pm 0.08$, $-0.61 \pm 0.06$, $-0.49 \pm 0.02$ or $-0.45 \pm 0.03$ for group Maculatin 1.1, Brevinin-1, Pseudin-2, ranatuerin-1, HP(2–20), CAP18, Tritrp1, MIIa or IsCT, respectively, indicating that $F$ is species-dependent for these AMP families. The wide distribution of $F$ value (Fig. 1) showed a flexibility diversity in AMPs derived from different species, possibly outlining a flexibility requirement for the diverse antimicrobial actions of the peptides [19–22]. By comparison, hydrophobicity $H$, hydrophobic moment $\mu H$ and positive charge density $\rho$ are also species-dependent and take mean values ranging from $-0.14$ to 0.74, from 0.27 to 0.79 and from 0.09 to 0.55, respectively (Table 1).

However, flexibility may be either species-specific for some AMP group pairings or not for others, like hydrophobicity and hydrophobic moment as well as positive charge density. In fact, by examining statistically differences of these four structural parameters, we found that, for all 36 different group pairings in the nine groups, flexibility index $F$ is species-specific ($p < 0.05$) except for four pairings (group MIIa and either ranatuerin-1 or pseudin-2, group Tritrp1 and either Maculatin 1.1 or Brevinin-1) (Table C in Appendices); by comparison, hydrophobicity moment $\mu H$ is not species-specific ($p > 0.05$) only for three group pairings (group CAP18 with either pseudin-2 or HP(2–20) or MIIa) (Table D in Appendices); the six group pairings with non-species-specific hydrophobicities ($p > 0.05$) are group ranatuerin-1 and MIIa, group IsCT and either Maculatin 1.1 or Brevinin-1, and group Tritrp1 and either Maculatin 1.1 or Brevinin-1 or IsCT (Table E in Appendices); and, charge density $\rho$ is not species-dependent ($p > 0.05$) only for eight group pairings, including group MIIa with IsCT, group Pseudin-2 with either Brevinin-1 or MIIa or IsCT, and group ranatuerin-1with each of group Brevinin-1, Pseudin-2, MIIa and IsCT (Table F in Appendices). These data indicated that, significant differences lie at least in one of these structural parameters for each group pairing, dictating that these structural parameters together have the ability to distinguish different AMP groups from each other; and except $\mu H$, species-dependent difference of $F$ is more significant than those of both $H$ and $\rho$, suggesting that flexibility index $F$ may serve as another antimicrobial determinant different from others.

### 3.2. Flexibility index reflects the mechanical properties of the peptides

Based on the distribution of flexibility index $F$ (Fig. 1), we herein divide all the 78 peptides into three clusters, the rigid one with $F < -0.54$, the flexible one with $F > -0.44$ and the moderate rigid or flexible one with $F$ within region from $-0.54$ to $-0.44$. With this

**Table 1**
The antimicrobial activity index $MIC^{-1}$, flexibility index $F$, hydrophobicity $H$, hydrophobic moment $\mu H$, charge density $\rho$ and alignment scores of nine AMP groups.

| Group name | $MIC^{-1}$ (ìM$^{-1}$) | $F$ | $H$ | $\mu H$ | $\rho$ | Alignment Score |
|---|---|---|---|---|---|---|
| Maculatin1.1 | 0.06 ± 0.04 | −0.66 ± 0.04 | 0.66 ± 0.03 | 0.46 ± 0.02 | 0.09 ± 0.04 | 95.00 ± 2.50 |
| Brevinin-1 | 0.12 ± 0.10 | −0.57 ± 0.01 | 0.76 ± 0.03 | 0.36 ± 0.04 | 0.16 ± 0.02 | 86.29 ± 9.84 |
| Pseudin-2 | 0.09 ± 0.07 | −0.51 ± 0.02 | 0.38 ± 0.04 | 0.57 ± 0.03 | 0.18 ± 0.07 | 92.14 ± 5.30 |
| ranatuerin-1 | 0.07 ± 0.05 | −0.48 ± 0.03 | 0.44 ± 0.07 | 0.27 ± 0.03 | 0.18 ± 0.03 | 90.25 ± 5.46 |
| HP(2–20) | 0.30 ± 0.17 | −0.36 ± 0.05 | 0.17 ± 0.19 | 0.62 ± 0.07 | 0.37 ± 0.00 | 88.83 ± 6.65 |
| CAP18 | 0.55 ± 0.74 | −0.23 ± 0.08 | −0.14 ± 0.14 | 0.59 ± 0.11 | 0.55 ± 0.06 | 92.00 ± 2.74 |
| Tritrp1 | 0.10 ± 0.04 | −0.61 ± 0.07 | 0.74 ± 0.01 | 0.41 ± 0.03 | 0.31 ± 0.00 | 81.86 ± 8.65 |
| MIIa | 0.65 ± 0.56 | −0.49 ± 0.02 | 0.44 ± 0.07 | 0.53 ± 0.04 | 0.20 ± 0.05 | 61.40 ± 13.0 |
| IsCT | 0.31 ± 0.23 | −0.45 ± 0.03 | 0.72 ± 0.06 | 0.79 ± 0.05 | 0.22 ± 0.08 | 85.60 ± 8.76 |

The data were presented by means plus stds of the involved peptides in each group, and the flexibility index $F$ was evaluated by $F_3$ (Table B).

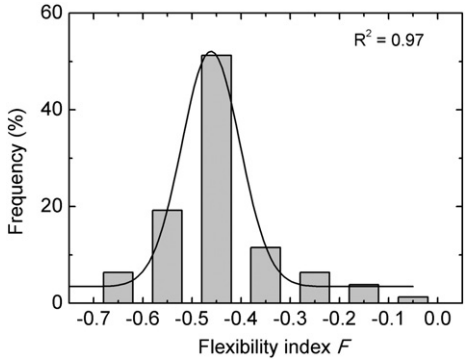


**Fig. 1.** The distribution of flexibility indices *F* of seventy-eight AMPs. The flexibility indices data of these peptides (Table A and B in Appendices) were fitted to the Gaussian distribution (solid line) with mean of −0.46 and standard derivation of 0.12.

definition, the AMPs in group Maculatin 1.1, Brevinin-1 and Tritrp1 were rigid, the group HP(2–20) and CAP18 were flexible, and the group Pseudin-2, ranatuerin-1, MIIa and IsCT were the moderate rigid or flexible (Table 1).

Obviously, the larger the flexibility index *F* is, the softer the peptide should be, hinting a monotonous relation between *F* and Young's modulus *E* for the clustered peptides. To test this, we plotted Young's moduli against the flexibility indices of the peptides in group HP(2–20) (Fig. 2), where the data of Young's moduli were taken from our previous work [24]. As predicted, Young's modulus decreases linearly with the increase of flexibility index, showing that the flexibility index does reflect the mechanical features of the linear cationic α-helical peptide HP(2–20) and its four analogs except HPA2. Little difference in the flexibility indices led to a significant variation in Young's moduli of peptide HPA1 and HPA2, and HPA1 has stronger resistance to tensile extension than HPA2 (Fig. 2), probably coming from that the α-helical amphipathic structure of HP(2–20) had been improved by substituting tryptophan for $Gln^{16}$ but not for $Asp^{18}$ in the peptide. It hints that the residue flexibility profiles should contribute to their respective mechanical properties of the peptide chains.

This results illustrated that the mechanical properties of the peptides in group HP(2–20) can be evaluated either by the molecular flexibilities relevant to B-factor or through spring constants derived by steered molecular dynamics simulation [24]. Probably, the linear relation between Young's modulus and flexibility index may exist in other peptide groups, because the conformations of their involved peptides are similar to those in group HP(2–20). In fact, all the 78 peptides are cationic and amphipathic due to their positive charge numbers and nonzero hydrophobicity moments. Besides the high sequence similarities between native peptides and their respective

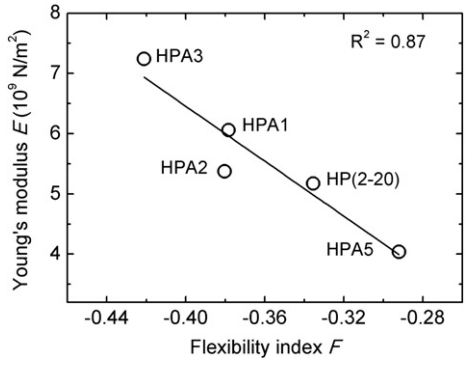


**Fig. 2.** Variation of Young's modulus against flexibility index *F* for AMP HP(2–20), HPA1, HPA2 HPA3 and HPA5. The Young's moduli were estimated through steered molecular dynamics simulations [24]. The best fitting of the data showed a linear relationship between Young's modulus and flexibility index (solid line).

analogs (Table 1; Table B in Appendices), an α-helical conformation was adopted by each peptides in group Maculatin 1.1 [31], pseudin-2 [33], ranatuerin-1 [34], HP(2–20) [23] and MIIa [36]; the peptides in both group CAP18 [35] and IsCT [37] also maintain their α-helical conformations except C18P and [$A^6$]-IsCT; and, Tritrp1 and its analogs retained the turn-turn or extended α-helical structures [30]. So, the peptides in each AMP group are homogenous, and drastic conformational changes might rarely occurred in the homogenous peptides.

Besides tensile property, the flexural rigidity $\kappa_f$ should be reflected also by flexibility index *F*. Supposing that the fitting relation of *E* and *F* for group H(2–20) (Fig. 2) is also obeyed by other peptides, we calculated persistence length $\xi_p$, a scale of flexural rigidity $\kappa_f$, through modeling each peptide as a circular uniform rod of radius of 0.25 nm [24] (Materials and methods). All results were summarized in Table 2. As expected, each peptide in the nine AMP groups is found to be rod-like for its persistence length $\xi_p$ (>8 nm) much longer than its contour length $L_c$ (<4 nm), except the peptide C18P ($\xi_p$ = 2.55 nm) and C18K ($\xi_p$ = 1.25 nm). It demonstrated that these peptides have enough bending resistances to maintain conformation stability, as binding to target cell membrane. And, the data (Tables 1 and 2) demonstrated that slightly decreasing of *F* will lead to strongly increasing of Young's modulus *E*; as a result, the Young's moduli of the rigid peptides with $F < -0.54$ are larger than about $10^{10}$ N/m$^2$, almost the two to four folds of the flexible peptides with $F > -0.54$, indicating that the group Maculatin 1.1, Brevinin-1 and Tritrp1 is more rigid in comparison with the group HP(2–20) and CAP18. This is in good agreement with the mentioned definitions for rigid and flexible peptides.

### 3.3. Various relationships between flexibility and other structural properties

It was thought that flexibility is different from but relevant to other structural properties, such as hydrophobicity and hydrophobic moment as well as charge density. For example, an increase of hydrophobicity may accompany with a decrease of flexibility, because of that, of all eight hydrophobic amino acid residues, only the Pro residue belongs to ten flexible residues (Gly, Thr, Arg, Ser, Asn, Gln, Asp, Pro, Glu and Lys) [29]. However, in the nine AMP groups, the flexibility should possess different relations not only with hydrophobicity but also with hydrophobic moment and charge density due to the various amino acid residue substitution strategies involved in these groups, even though the peptides in each group except group MIIa are homogenous (Table 1; Table B in Appendices). To examine it, we plotted hydrophobicity ($H$), hydrophobic moment ($\mu H$) and charge density ($\rho$) against flexibility index ($F$) for each AMP groups (Fig. 3A, B and C).

We found that flexibility is linearly dependent on hydrophobicity for flexible peptides rather than for rigid peptides. The besom-like *H-F* pattern (Fig. 3A) exhibited that, overall, as flexibility index *F* decreased, hydrophobicity *H* increased monotonously first until *F*

**Table 2**
The elastic moduli, persistence lengths and the mean contour lengths of free rod-like peptides.

| Group name | $E$ ($10^9$ N/m$^2$) | $\xi_p$ (nm) | $L_c$ (nm) |
|---|---|---|---|
| Maculatin1.1 | 12.37 ± 0.85 | 38.84 ± 2.70 | 3.35 |
| Brevinin-1 | 10.32 ± 0.18 | 32.66 ± 0.58 | 3.83 |
| Pseudin-2 | 8.90 ± 0.46 | 28.15 ± 1.45 | 3.83 |
| ranatuerin-1 | 8.37 ± 0.70 | 26.49 ± 2.22 | 3.99 |
| HP(2–20) | 5.57 ± 1.18 | 17.62 ± 3.72 | 3.03 |
| CAP18 | 2.55 ± 1.60 | 8.07 ± 5.05 | 3.19 |
| Tritrp1 | 11.15 ± 1.38 | 35.28 ± 4.37 | 2.07 |
| MIIa | 8.48 ± 0.37 | 26.85 ± 1.17 | 3.67 |
| IsCT | 7.50 ± 0.55 | 23.75 ± 1.75 | 2.07 |

Here, $E$, $\xi_p$, and $L_c$ are the elastic modulus, persistence length, mean contour length of free-peptides, respectively. All data were presented by means plus stds.

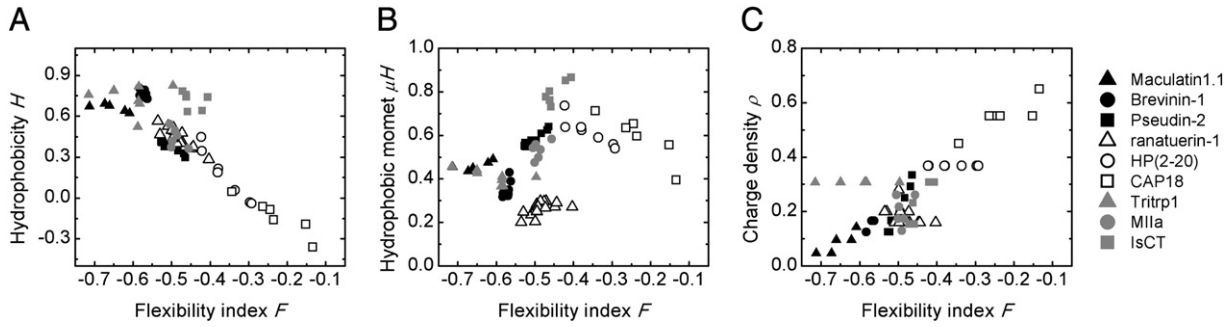


**Fig. 3.** Variations of hydrophobicity $H$ (A), hydrophobic moment $\mu H$ (B), and charge density $\rho$ (C) against flexibility index $F$. The data referred to all peptides in the nine AMP groups, such as group Maculatin 1.1(●, dark), Brevinin-1(▲, dark), pseudin-2 (■, dark), ranatuerin-1(○, dark), HP(2–20) (△, dark), CAP18 (□, dark), Tritrp1 (●, gray) and MIIa (▲, gray) as well as the group IsCT (■, gray).

reached a value of about −0.4 and then slid into different branches for the nine peptide groups. The monotonous dependence of hydrophobicity on flexibility existed in flexible AMP group HP(2–20) and CAP18, but the *H*-*F* pattern was bi-phasic for group Maculatin 1.1 and IsCT, tri-phasic for group pseudin-2 and irregular for group Tritrp1, Brevinin-1Bb and MIIa. And for group ranatuerin-1, hydrophobicity $H$ was decreased along two parallel descending pathways with increase of $F$ (Fig. 3A). Unlike hydrophobicity $H$, hydrophobic moment $\mu H$ irregularly varied with flexibility on the whole for the nine AMP groups (Fig. 3B). Even so, as flexibility index $F$ increased, hydrophobic moment $\mu H$ seemed to decrease for group HP(2–20) and CAP18 but increase for group Maculatin 1.1 and pseudin-2, or be noncommittal for other AMP groups. Likewise, charge density ($\rho$) maintained a constant level in group HP(2–20) and Tritrp1, and rose either step by step for group Maculatin 1.1 and IsCT or to a higher plateau for group CAP18 as $F$ increased, but varied non-monotonically with $F$ for other AMP groups. A flexible AMP usually had a higher charge density than those of rigid AMP groups, even though the $\rho$ -$F$ patterns were diversified remarkably for the nine AMP groups (Fig. 3C). These data showed that mutation-induced changes of relations between flexibility and other structural properties might be dramatic.

The overall correlation coefficients (CCs) between $F$ and each of $H$, $\mu H$ and $\rho$ within each group were summarized in Table 3. These data showed that, flexibility is related significantly to hydrophobic moment either inversely for group Tritrp1, HP(2–20) and CAP18 ($CCs \leq -0.64$) or directly for other groups ($CCs \geq 0.54$), and inversely to hydrophobicity $H$ either unconspicuously for group Tritrp1 ($CC = -0.02$) or strongly for group Pseudin-2, HP(2–20) and CAP18 ($CCs \leq -0.94$) or significantly for other groups ($CCs \leq -0.33$); and, except group HP(2–20) and Tritrp1, positive charge density $\rho$ is relevant to flexibility index $F$ inversely for group Pseudin-2 ($CC = -0.23$) but directly for other groups ($CCs \geq 0.24$). And, the correlation coefficients of $F$ to $H$, $\mu H$ and $\rho$ varies from −0.51 to −0.98, from −0.9 to 0.96 and from −0.23 to 0.87, respectively, for the first six AMP groups (Table 3), even though there is a high sequence identity within each of these groups (Table 1). It suggested a species-dependent correlation among these structural parameters, as expected.

**Table 3**
The correlation coefficients between flexibility index $F$ and hydrophobicity $H$, hydrophobic moment $\mu H$ or charge density $\rho$ of peptides in each group.

| Group name | Correlation coefficient | | |
|---|---|---|---|
| | $F$ vs $H$ | $F$ vs $\mu H$ | $F$ vs $\rho$ |
| Maculatin1.1 | −0.81 | 0.75 | 0.87 |
| Brevinin-1 | −0.51 | 0.55 | 0.24 |
| Pseudin-2 | −0.95 | 0.96 | −0.23 |
| ranatuerin-1 | −0.84 | 0.54 | 0.61 |
| HP(2–20) | −0.98 | −0.90 | N/A |
| CAP18 | −0.94 | −0.89 | 0.86 |
| Tritrp1 | −0.02 | −0.64 | N/A |
| MIIa | −0.53 | 0.57 | 0.26 |
| IsCT | −0.33 | 0.85 | 0.90 |

### *3.4. Flexibility may be a mechanical determinant for antimicrobial activities of peptides*

To investigate whether flexibility is a mechanical determinant for antimicrobial activities of AMPs or not, we examined the variation of activity index (the reciprocal of the minimum inhibition concentration aimed at *E. coli*) with flexibility index $F$ in nine AMP groups (Fig. 4A and B). A flexibility-dependent antimicrobial activity was exhibited in Fig. 4A for six AMP groups, including group Maculatin 1.1, Brevinin-1, Pseudin-2, ranatuerin-1, HP(2–20) and CAP18. As $F$ increased, the activity index would exponentially increase for both rigid group Maculatin 1.1 and Brevinin-1, but decrease exponentially for both flexible group HP(2–20) and CAP18; the functional transition of flexibility would occur in the moderate hard group Pseudin-2 or ranatuerin-1, as the activity index varied with $F$ (Fig. 4A, Fig. A in Appendices). In trend, the activity index increased with $F$ first and then followed a "pseudoplateau" over a narrow band of $F$ after passing a inflection point of $F$ with values of about −0.5 for group Pseudin-2 (Fig. 4A, Fig. A.1 in Appendices); on the contrary, the activity index remained in the "pseudoplateau" as $F$ increased from −0.536 to −0.5 first, and then decreased as $F$ increased further for group ranatuerin-1(Fig. 4A, Fig. A.2 in Appendices).

These data indicated that, for the more flexible peptides in group ranatuerin-1, HP(2–20) and CAP18, flexibility seems to be a reducer of the antimicrobial activity, probably referring to the rigidity-enhanced antimicrobial actions, as described in our previous work for group HP(2–20) [24]; on the contrary, flexibility may act as an enhancer of the antimicrobial activity for the rigid peptides in group Maculatin 1.1, Brevinin-1 and Pseudin-2, possibly exhibiting a requirement on a balance between structural stability and deformability of these peptides in interacting with and exploiting vulnerabilities inherent in the target cell membrane [24].

However, the above flexibility-dependent antimicrobial activity vanished in group Tritrp1, MIIa and IsCT (Fig. 4B). The reason may come either from the lower sequence identity (<73%) within group MIIa or from the shorter residue chain length (=13) of peptides in group Tritrp1 and IsCT, in comparison to other six groups (Table 1). We found no trend change in plots of $MIC^{-1}$ against $F$, while the data derived from peptides with sequence alignment scores <85 were removed from Fig. 4 for each group (Data not shown). It suggests that high sequence identity is necessary but not enough, in scaling antimicrobial activity with flexibility. And, for the peptides in group Tritrp1 and IsCT, their residue chain lengths are so short that residue substitutions dramatically change conformations and physical properties of those mutations. It was demonstrated by the loop-like and extended α-helical structures of peptides (Tritrp7 and

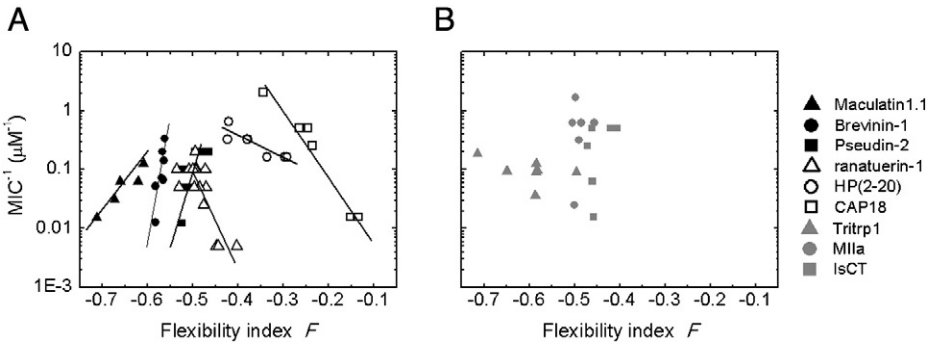


**Fig. 4.** Variation of reciprocal of MIC against flexibility index *F* for the nine AMP groups. (A) Two-phase flexibility-dependent antimicrobial activity in six AMP groups, including group Maculatin 1.1, Brevinin-1, Pseudin-2, ranatuerin-1, HP(2–20) and CAP18. As the flexibility index *F* increases, the antimicrobial activity either increases for group Maculatin 1.1, Brevinin-1 and Pseudin-2 or decreases for group ranatuerin-1, HP(2–20) and CAP18. (B) The untidy correlations between the flexibility and the antimicrobial activity in group Tritrp1, MIIa and IsCT.

Tritrp8) with sequence identity of 92% (Fig. B and Table B in Appendices) [30], and also reflected by the complex correlations between *F* and either *H* or *μH*, as shown in the "*T*"-shaped *F*–*H* profile for group Tritrp1, the "*U*"-shaped *F*–*H* profile and the "<"-shaped *F*–*μH* profile for group IsCT (Fig. 3A and B). These dramatic changes should be more important than flexibility alone in dictating functions of these short peptides. Meanwhile, the short chain length might decrease reliability in calculating flexibility index, because the residue flexibility indices were derived from proteins of sequence lengths of hundreds [29]. Besides, amphipathicity structure may be another requirement for the flexibility-dependent antimicrobial activity. For example, amphipathic structures surely appeared not only in CAP18 but also in HP(2–20) and its analogs (Fig. 5A and Fig. B.1, white); by contrast, there was a considerable region encircled by the hydrophobic residues in Tritrp1, IsCT and their analogs (Fig. 5B and Fig. B.2–3), the locally losing of amphipathicity of the α-helical structure would enhance peptide dimerization [15,42], which might remarkably change antimicrobial actions of the peptides.

## 4. Discussion

Flexibility was mentioned mainly as a structural property to govern folding, stability and function of protein [43,44]. Here, we showed that, flexibility reflects the flexural resistance and may act as a mechanical determinant of antimicrobial actions for a linear cationic amphipathic α-helical AMP. Increasing flexibility may lead the antimicrobial activity to be either decreased for a soft peptide or increased for a hard one (Fig. 4A). This flexibility-dependent antimicrobial activity may refer to a mechanical mechanism of antimicrobial actions, such as slot or pore formation [1,12,24].

Firstly, the initial nonspecific interactions of antimicrobial peptides with the target cell make the cellular membrane either free of or associated with the peptides, and the formation of peptide-bound leaflets in the membrane ensues [45,46]. Then, the augmented bending resistances of the peptide-bound leaflets will create internal membrane tensions, resulting in transient or prolonged stress concentrations at edges of the peptide-bound leaflets and further facilitate the slot or pore formation in the membrane under active or passive movements [24,47]. The harder the bound peptides are, the more significant the local stress concentrations in the undulated membrane will be, and the more easily the slots or pores in the membrane will form, because of a more remarkable bending rigidity leap from peptide-free to peptide-bound leaflets [24,48,49]. This rigidity requirement may be an explanation on the rigid-enhanced or flexibility-reduced antimicrobial activity for flexible peptides such as in group HP(2–20), ranatuerin-1 and CAP18 (Fig. 4A), as described in our previous work for group HP(2–20) [24].

However, the peptides should not be too hard for their realization of antimicrobial activity, because stress concentration requires formation of stable peptide-bound leaflets. In comparison to a hard peptide, a limp one has a better structural compliance so that the peptide can more smoothly spread on and closely associate with membrane to form a stable peptide-bound leaflet. Mechanically, in forming a stable peptide-bound peptide, the hydrophobic and electrostatic driving forces will bend a straight peptide to match with cellular membrane. Under these forces, this limited bending deformation realizes easily for a flexible peptide but does not for a rigid one. Meanwhile, the poor positive charges on a too hard peptide (Fig. 3C) are unfavorable for forming a stable peptide-bound leaflet. As a result, if the bound-peptide is too hard, there might just have a slight bending rigidity leap at the edge of the peptide-bound leaflet, because the bending resistance of the bound-peptide leaflet might drop down steeply through partially sliding of the loosely bound-peptide on membrane. Thus, there may have a balance between two contrary effects of flexibility on antimicrobial actions, one weakens stress concentrations, and other prompts formation of stable peptide-bound leaflet. It is from this balance that there may be an optimum flexibility index of about −0.5, across which flexibility switches from an enhancer to a reducer of antimicrobial actions (Fig. 4A). Below the optimum, increasing flexibility contributes mainly to increasing flexural deformation in favor of subsequent stable peptide-bound leaflet formation, suggesting a flexibility-enhanced antimicrobial activity mediated mainly through flexibility-prompted formation of stable peptide-bound leaflet for

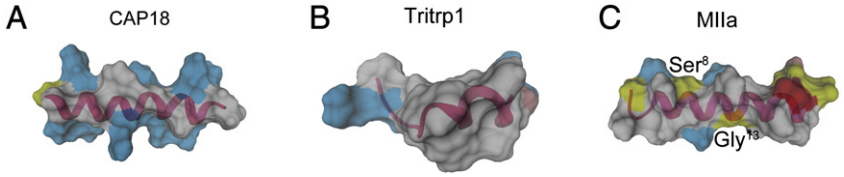


**Fig. 5.** Representative MSMS structures of some AMPs. A, CAP18; B, Tritrp1; C, MIIa. The secondary structures were showed by Newcartoon representation (transparent, pink), and the residues properties were colored as follows: hydrophobic (white), basic (blue), acidic (red) and solvent (yellow). The PDB files were downloaded from RCSB Protein Data Bank (http://www.pdb.org/pdb/home/home.do) with the PDB codes: 1LYP (CAP18), 2I1D (Tritrp1), and 2MAG (MIIa). CAP18 was a fragment of the structure recorded in 1LYP.

rigid peptides such as in group Maculatin 1.1, Brevinin-1 or pseudin-2. This may also explain why the flexible hinge structure leads to high activities of some AMPs aimed at their target cell [19–22]. In contrast, increasing flexibility above the optimum has an insensitive effect on bending deformation of peptide-bound leaflet due to the small bending resistance, but remarkably decreases the bending rigidity leap at the edge of peptide-bound leaflet, suggesting a flexibility-reduced antimicrobial activity induced by rigidity-prompted stress concentration for flexible peptides such as in group HP(2–20), ranatuerin-1 or CAP18.

Besides the peptide flexibility or rigidity, the mechanical properties of cellular membrane should be relevant to the above antimicrobial processes. Through weakening the stress concentration in the membrane bound with peptides, increasing of bending rigidity of cellular membrane will make the peptide-induced crash of the membrane difficult. Thus, the bending rigidities of bound peptide and cellular membrane may act as "mechanical impedances". The matching between the bending rigidities of peptide and membrane will yield an optimum value of peptide flexibility or rigidity. At this optimum, the peptide has the maximum antimicrobial activity, because the most significant stress concentration may occur at the edge region of peptide-bound leaflet. As mentioned above, flexibility can be related to persistence length through Young's modulus (Table 2) for the rod-like peptides. Except peptide C18P and C18K with persistence length ($\xi_p$) <2.55 nm, each peptide in the nine AMP groups may be stable and rod-like for its long persistence length (>8.53 nm) in comparison with its contour length $L_c$ (<4 nm). At optimum point of flexibility index, the relative persistence length $\xi_p/L_c$ takes a value of about 6.84 (Table 2).

Unlike flexibility, the effects of hydrophobicity *H*, hydrophobic moment $\mu H$ and charge density $\rho$ on antimicrobial activity (1/MIC) were found herein mostly to be ambiguous for the nine AMP groups (Fig. C and D in Appendices), as demonstrated in the previous reports [14,15,30]. For example, 1/MIC increased with *H* only in Group ranatuerin-1 and CAP18 (Fig. C 4 and 6 in Appendices), and likewise, decreases with $\mu H$ only in group CAP18 and IsCT (Fig. C 6 and 9 in Appendices). These significant differences in effects of *F* and other structural parameters on 1/MIC should be relevant to the diverse relationships of *F* to other structural parameters (Fig. 3). In fact, the species-dependent branching in the besom-like *H*-*F* pattern (Fig. 3A) showed a multivalve dependence of *H* on *F*, and would make the relationship of 1/MIC with *H* more complex than with *F* (Fig. 4A, Fig. C). It made a barrier in uncovering a relationship of 1/MIC with these structural parameters through multivariate analyses. Even so, the overall correlations of ln(1/MIC) to *F*, *H*, $\mu H$ and $\rho$ showed that *F* correlates with ln(1/MIC) more strongly than other structural parameters for each peptide groups except for two or three groups (the group ranatuerin-1, Tritrp1 and MIIa for *H*; the group Tritrp1, MIIa and IsCT for $\mu H$; MIIa and IsCT for $\rho$) (Table G in Appendices), suggesting that flexibility index may be a better structural parameter than others in scaling the antimicrobial activity.

However, high sequence similarities within the peptides in the first six AMP groups suggest a high sequence identity requirement in scaling antimicrobial activity with flexibility (Table 1; Fig. 4A). This requirement is necessary but not enough, because flexibility does not function as either an enhancer or a reducer of antimicrobial activity not only for the low homogenous group MIIa (sequence identity < 73%) but also for the high homogenous members (sequence identity > 85%) in group IsCT and Tritrip 1. Another requirement may be that the peptides should not be too short. An over short chain length decreases reliability of the calculated flexibility, and may make the mutation-induced changes of both conformations and overall structural properties dramatic. For example, even though the persistence lengths (>30 nm) of the peptides in group Tritrp1 are much longer than their corresponding contour lengths (<4 nm), the loop-like and extended α-helical structures of the peptides seem to be flexible (Fig. B.2) [30], and may undergo a conformational transition from a looser state to either a more stable α-helix under structure-induced driving force on the membrane interface or not [13], dictating a limitation of flexibility index in scaling function of short peptides; and, for each peptide in group IsCT with a considerable region encircled by the hydrophobic residues (Fig. B.3), locally losing of amphipathicity in α-helical structure makes hydrophobic face discontinuous and may enhance peptide dimerization [15,42], which will make the effect of flexibility on antimicrobial activity distinct.

Summarily, flexibility index, as a scale for tensile or flexural rigidity of α-helical peptides, can be used to describe structural stability and deformability of the peptides, and regarded as a mechanical determinant in antimicrobial actions, such as the peptide-bound formation and the stress concentrations in target cell membrane. It was demonstrated that flexibility also acts on many other biological reactions. For example, high stability or low flexibility may lead to the loss of activity [50]; the proteolytic enzyme MMP-12 is more active than MMP-3, because MMP-12 is more flexible [18]; and, a mutation of the optimized metallo-β-lactamase gained later in the evolution further enhances the catalytic efficiency by augmenting the flexibility [51]. And, the species-dependence of flexibility may be required in the antimicrobial function realization of the peptides. But, for the interdependent structural properties, a modification of one often accompanies with a compensatory alterations in others, so that the antimicrobial activity cannot be scaled generally just by a single structural parameter. It limits the potential of flexibility index in scaling antimicrobial activity, especially for the peptides short over, even though these peptides are highly homogenous. This also is why it is still a challenge to reveal the structure–activity relations of AMPs. However, for a linear cationic amphipathic α-helical peptide, its flexibility may be a dominant activity determinant, and should be useful in seeking for a new structure–activity relationship. Besides, the present work provided a new mechanical viewpoint in understanding antimicrobial actions and might be useful in developing a novel amino acid residue substitute strategy in flexibility-dependent antimicrobial activity design of α-helical peptides.

## Acknowledgments

This work was supported by NSFC grants 10772069, 11072080 (JW), and 10972081 (YF) and by Guangdong Science and Technology Projects of Industrial Research grant B011000017 (JW).

## Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamem.2013.06.017.

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