Hydrophobicity, hydrophobic moment and angle subtended by charged residues modulate antibacterial and haemolytic activity of amphipathic helical peptides

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Abstract The hydrophobicity (H), hydrophobic moment (μ) and the angle subtended by the positively charged helix face (Φ) of a set of model and magainin 2 amide peptides with conserved charge and helix propensity have been shown to be effective modulators of antibacterial and haemolytic activity. Except peptides of low hydrophobicity which are inactive, changing the parameters has little influence on the activity against Gram-negative bacteria, thus revealing the dominance of electrostatic interactions for the effect. However, the increase of H, μ and Φ substantially enhances haemolytic and Gram-positive antibacterial activity and is related to a reduction of peptide specificity for Gram-negative bacteria.

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Key words: Antimicrobial peptide; Haemolysis; Hydrophobicity; Hydrophobic moment; Magainin

1. Introduction

During the last decade a multitude of host defence peptides have been found in a wide range of species (for review, see [1–3]). These peptides can broadly be classified into cytolytic peptides, active against prokaryotic as well as eukaryotic cells such as melittin and antimicrobial peptides such as the magainins. Both classes of peptides exert their activity by enhancing the permeability of the cell membrane which has been suggested to be caused by the formation of ion channels or a global disturbance of the lipid bilayer [4,3]. Because of their putative clinical relevance, structure–function correlation of membrane active peptides has been the subject of extensive investigations [5–9]. Recent studies have correlated the specific antimicrobial activity of the magainins with the high content of negatively charged phospholipids, the inside negative transmembrane potential and the low cholesterol content of bacterial cell membranes [10–12]. However, despite advances in identifying activity modulating membrane properties, the structural differences between antimicrobial and lytic peptides are subtle. Generally, membrane active peptides are able to form an amphipathic secondary structure in a membrane environment. Significantly, in addition to assuming mostly an α-helical conformation the antibacterial peptides are cationic in nature. The positive charge was found to drive peptide binding at negatively charged lipid bilayers [13–15] and can also be

expected to facilitate peptide attachment at the negatively charged bacterial surface [16].

Several attempts have been described to improve antimicrobial activity and enhance selectivity by increasing the positive charge and/or helicity [17,18,5,19]. However, often enhanced activity was found to be connected with reduced selectivity. More recent investigations using a cationic amphipathic model peptide [14] and paradaxin analogs [20] revealed that helicity is less crucial for the antibacterial effect. However, the correlation of helicity of lipid membrane bound peptides with haemolytic activity supported the significant role of the amphipathic helix for the effect on red blood cells. The investigations led to the conclusion that peptide modifications that enhance the disturbance of the negatively charged phospholipid headgroups should increase antibacterial activity whereas modifications compatible with an increase in hydrophobic peptide–membrane interactions should enhance the haemolytic effect. Actually, changes in the total hydrophobicity of the model peptide have been found to correlate with the ability to permeabilize electrically neutral model membranes whereas the influence on activity observed at negatively charged lipid bilayers could be neglected [14]. Additionally, studies with magainin analogs revealed that the selectivity for negatively charged membranes substantially decreases with increasing peptide hydrophobicity [21]. These results and recent investigations of Maloy et al. [5] and Zhong et al. [6] prompted us to examine the effect of hydrophobicity (H), the hydrophobic moment (μ) and the angle (Φ) subtended by the cationic helix domain on membrane activity and selectivity. Using a set of potentially helical model peptides and magainin 2 amide (M2a) analogs, these structural properties were related in this study to antibacterial and haemolytic activity.

2. Materials and methods

Model peptides and M2a analogs (Table 1) were synthesized automatically by the solid-phase method using standard Fmoc chemistry in the continuous flow mode [22]. The peptides were purified by preparative high performance liquid chromatography (HPLC) to give final products more than 95% pure by RP-HPLC analysis and characterized by quantitative amino acid analysis. Trifluoroethanol (TFE) was obtained from Aldrich-Chemie (Steinheim, Germany) and the other chemicals were from Merck (Darmstadt, Germany).

Peptide hydrophobicity (H) and the hydrophobic moment (μ) for an ideal helix were calculated using the Eisenberg's scale for hydrophobicity and moment method [23].

Circular dichroism (CD) measurements of 10^{-5} M peptide solutions in TFE/buffer (10 mM Tris(hydroxymethyl)aminomethane, 154 mM NaF (pH 7.4), 1:1 (v/v)) were carried out on a J720 spectrometer

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(Jasco, Japan) between 185 and 260 nm at room temperature. The helicity was determined from the mean residue ellipticity at 222 nm according to Chen et al. [24]. The error in the helicity was 5%.

The haemolytic activity of the peptides was determined using human red blood cells as described previously [14]. In brief, 150 μl of cell suspension containing 1.2×10^9 cells/ml and varying amounts of peptide stock solution (concentration usually 10^{-4} mol/l in Tris buffer) and buffer were pipetted into Eppendorf tubes to give a final volume of 1000 μl. The suspensions containing 1.8×10^8 cells/ml were incubated for 30 min at 37°C. After cooling in ice water and centrifugation at $2000 \times g$ for 5 min, 200 μl of supernatant was combined with 1800 μl of 0.5% NH_4OH and the optical density was measured at 540 nm. Peptide concentrations causing 50% haemolysis (EC_{50}) were derived from the dose–response curves. Values determined in repeat experiments differed by less than 5%.

Antimicrobial susceptibility testing was performed using a modification of the National Committee for Clinical Laboratory Standards (NCCLS) microdilution broth assay [25]. Mueller Hinton broth (BBL, Cockeysville, MD) was used for diluting the peptide stock solution and the bacterial inoculum. The inoculum was prepared from mid logarithmic phase cultures at an approximate concentration of 1×10^6 CFUs/ml and 100 μl of this solution was added to 100 μl of the peptide dilution in the well. The final concentration of peptide solution ranged from 0.25 to 256 μg/ml in 2-fold dilutions. The final concentration of bacteria in the wells was 1×10^5 to 5×10^5 CFUs/ml. Peptides were tested in duplicates. In addition to the test peptide, three standard peptides and a non-treated growth control were included to validate the assay. The microtiter plates were incubated overnight at 37°C and the absorbance was read at 630 nm. The Minimal Inhibition Concentration (MIC) is defined as the lowest concentration of peptide that completely inhibits growth of the test organism.

3. Results

The 18-residue model peptide (KLA1) and M2a consisting of 23 amino acid residues used as basis for structural modifications exhibit significant differences in their structural motifs (Fig. 1, Tables 1 and 2). While KLA1 contains five lysine residues causing a net positive charge of +6, M2a bears four lysine, one histidine and one glutamic acid residue resulting in a net positive charge of about +4 at physiological pH. The H and μ of the model peptide KLA1 are comparably high (−0.025 and 0.33, respectively) and the angle subtended by the lysine residues (Φ) is low (80°), while H and μ of M2a are

lower (−0.0357 and 0.238, respectively) and Φ is significantly larger (120°). By minimal amino acid substitution (Table 1) based on the hydrophobicity scale of Eisenberg [22] and the conformational parameters for α-helical residues of Chou and Fasman [26] one specific peptide property such as hydrophobicity, hydrophobic moment and angle of the positively charged face of the amphipathic peptide helix was modified while conserving the other structural features (Table 2). The peptide charge was always kept constant and the helix propensity was retained (Table 2) as probed by CD measurements in structure inducing buffer/TFE mixture.

KLA1 displays both high antibacterial and haemolytic activity (Table 2). Reduction of H from −0.025 to −0.056 (KLA2) only slightly decreases the MIC against Gram-negative bacteria but causes substantial loss of activity against S. aureus and erythrocytes. Very low hydrophobicity (KLA3) abolishes activity. Changes of the hydrophobic moment are most effective at low peptide hydrophobicity. Thus, reduction of μ by 0.045 (KLA1 vs. KLA11) or increase of μ by 0.122 (KLA1 vs. KLA7) have little influence on activity on bacteria and only the last modification (Δμ = 0.122) causes a 10-fold increase in haemolytic activity. A comparably slight increase in μ by 0.062 of the more hydrophilic peptide KLA2 (KLA2 vs. KLA12) also scarcely influences the MIC against Gram-negative bacteria but enhances the activity against S. aureus and the haemolytic effect by a factor of about 30 and 10, respectively. The reduction of activity caused by decreasing H can be compensated for by an increase of μ (compare KLA1, KLA2 and KLA12). The angle subtended by the cationic residues of the amphipathic peptide helix exhibits little influence on the cytotoxic activity against bacteria. However, the haemolytic activity significantly increases if Φ was greater than 80° (KLA9, KLA10, KLA8,). The effect is not caused by the change in μ (KLA1 vs. KLA9) as the comparison of the results with KLA1 and KLA11 reveals.

The effects of H, μ and Φ on activity modification are confirmed by M2a peptides. The high MIC and EC_{50} values for M2a compared to KLA1 confirm the importance of total hydrophobicity and hydrophobic moment to the biological effect. The difference in activity is most pronounced on S.

Table 1
Sequences of investigated KLA and M2a peptides

| Sequence																								Abbreviation
1				5					10					15					20					
K	L	A	L	K	L	A	L	K	A	W	K	A	A	L	K	L	A	NH₂						KLA1
K	L	A	L	K	A	A	L	K	A	W	K	A	A	A	K	L	A	NH₂						KLA2
K	L	A	L	K	A	A	A	K	A	W	K	A	A	A	K	A	A	NH₂						KLA3
K	A	I	A	K	S	I	L	K	W	I	K	S	I	A	K	A	I	NH₂						KLA7
K	A	L	A	A	L	L	K	K	W	A	K	L	L	A	A	L	K	NH₂						KLA8
K	L	L	A	K	A	A	L	K	W	L	L	K	A	L	K	A	A	NH₂						KLA9
K	A	L	K	K	L	L	A	K	W	L	A	A	A	K	A	L	L	NH₂						KLA10
K	I	T	L	K	L	A	I	K	A	W	K	L	A	L	K	A	A	NH₂						KLA11
K	A	L	A	K	A	L	A	K	L	W	K	A	L	A	K	A	A	NH₂						KLA12
G	I	G	K	F	L	H	S	A	K	K	F	G	K	A	F	V	G	E	I	M	N	S	NH₂	M2a
G	I	G	K	F	L	H	S	A	K	K	F	G	K	A	W	V	G	E	I	M	N	S	NH₂	W¹⁶-M2a
G	L	G	K	F	L	H	S	A	K	R	F	G	K	A	F	V	G	E	A	M	N	S	NH₂	L²R¹¹A²⁰-M2a
G	I	G	K	F	I	H	S	A	K	K	F	G	K	L	F	V	G	E	I	M	N	S	NH₂	I⁶L¹⁵-M2a
G	I	G	K	F	I	H	A	A	K	K	F	G	K	L	F	I	G	E	I	M	N	S	NH₂	I⁶A⁸L¹⁵I¹⁷-M2a
G	I	G	K	F	I	H	S	A	K	R	F	G	R	A	W	V	G	E	I	M	N	S	NH₂	I⁶R¹¹R¹⁴W¹⁶-M2a
G	I	G	K	F	I	H	S	V	K	K	W	G	K	T	F	I	G	E	I	M	N	S	NH₂	I⁶V⁹W¹²T¹⁵I¹⁷-M2a
G	I	A	K	F	G	K	A	A	A	H	F	G	K	K	W	V	G	E	L	M	N	S	NH₂	100-M2a
G	I	G	K	F	L	H	T	L	K	T	F	G	K	K	W	V	G	E	I	M	N	S	NH₂	140-M2a
G	I	G	H	F	L	H	K	V	K	S	F	G	K	S	W	I	G	E	I	M	N	S	NH₂	160-M2a

KLA1   M2a
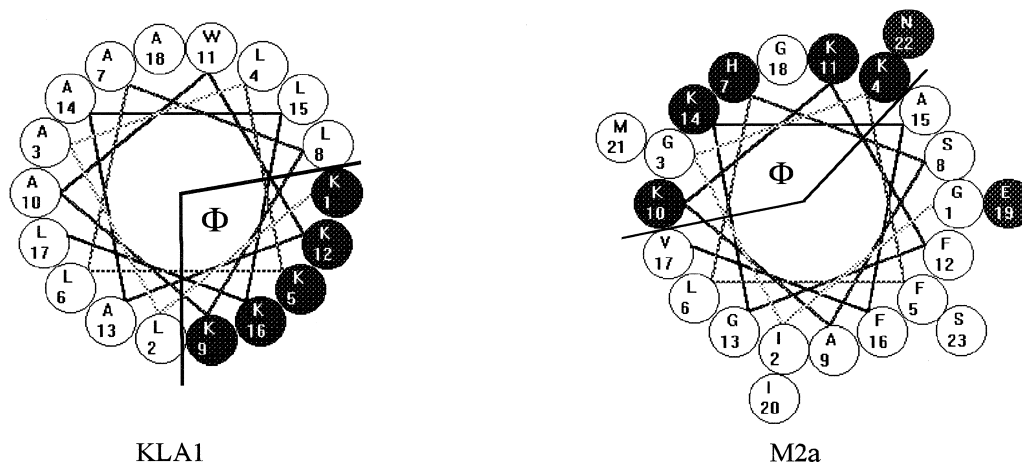
Fig. 1. Helical wheel projection of KLA1 and M2a.

aureus and on red blood cells where the MIC and EC_{50} values of the two peptides differ by a factor of about 40. The increase of H and μ for M2a enhances antibacterial as well as haemolytic activity. A higher hydrophobic moment ($I^6R^{11}R^{14}W^{16}$-M2a) even restores gone bioactivity to the most hydrophilic, biologically inactive peptide $L^2R^{11}A^{20}$-M2a. Changing the polar face of the magainin helix from 100° to 140° produced the most pronounced effect on the antibacterial activity, with the maximum activity observed for the 140-M2a analog.

With respect to selectivity of KLA and magainin peptides

the investigation revealed the following result: The differences between MIC and EC_{50} values for KLA peptides decrease with increasing H, μ and Φ. Reduction of the hydrophobicity of KLA1 (KLA1 vs. KLA2) increases the EC_{50} of haemolytic activity by a factor of 10 and an increase in the hydrophobic moment of KLA1 and KLA2 or an angle of the cationic face larger than 80° cause a 10-fold decrease of the EC_{50} values while the activity against *E. coli* and *P. aeruginosa* remains almost unchanged. Thus, low peptide hydrophobicity enhances Gram-negative bacterial specificity and large μ and Φ

Table 2
Structural parameters and antibacterial and haemolytic activity of KLA and M2a peptides

Variable	Peptide	H	μ	φ (°)	α (%) (50% TFE)	Antibacterial activity			Haemolytic activity
						E. coli MIC (μM)	*P. aeruginosa* MIC (μM)	*S. aureus* MIC (μM)	EC_{50} (μM)
H	KLA3	−0.087	0.329	80	59	91	>91	>91	>200
	KLA2	−0.056	0.329	80	68	5.5–11	45	45	107
	KLA1	−0.025	0.329	80	73	2.6–5.2	10.4–20.8	1.3–2.6	11
μ	KLA2	−0.056	0.329	80	68	5.5–11	44	44	107
	KLA12	−0.056	0.391	80	67	6	24	1.5	10
μ	KLA11	−0.026	0.284	80	69	5.3	21.2	5.3	10
	KLA1	−0.025	0.329	80	73	2.7–5.4	10.4–20.8	1.3–2.6	11
	KLA7	−0.026	0.451	80	70	5.6	11.2	1.4	1.8
φ	KLA1	−0.025	0.329	80	73	2.6–5.2	10.4–20.8	1.3–2.6	11
	KLA9	−0.025	0.295	120	55	6.2	12.4	1.6	1.7
	KLA10	−0.025	0.299	140	62	6.1	12.2	1.5	2.0
	KLA8	−0.025	0.291	180	62	2.9–5.8	5.8	3.0	2.5
H	$L^2R^{11}A^{20}$-M2a	−0.0957	0.287	120	45	75	>75	>75	>1000
	M2a	−0.0357	0.286	120	57	40	80	>80	428
	I^6L^{15}-M2a	−0.0148	0.284	120	57	19–38	76	38	260
	$I^6A^8L^{15}I^{17}$-M2a	0.0157	0.280	120	61	2.4	19–28	9.6	32
μ	$L^2R^{11}A^{20}$-M2a	−0.0957	0.287	120	45	75	>75	>75	>1000
	$I^6R^{11}R^{14}W^{16}$-M2a	−0.0983	0.332	120	52	37.5	75	>75	303
μ	M2a	−0.0357	0.286	120	57	40	80	>80	428
	$I^6V^9W^{12}T^{15}I^{17}$-M2a	−0.0352	0.317	120	64	2.3	18	18	56
φ	100-M2a	−0.045	0.288	100	48	75	>75	>75	700
	W^{16}-M2a	−0.046	0.288	120	57	40	80	>80	509
	140-M2a	−0.049	0.288	140	75	13	13	13	35
	160-M2a	−0.047	0.288	160	54	19	19	76	82

Peptide hydrophobicity per residue (H) was calculated from the sum of hydrophobicity values of the individual residues and the hydrophobic moment (μ) for an ideal helix was determined as described by Eisenberg [22].
Φ represents the angle subtended by the cationic residues on the ideal peptide helix.
The helicity (α) in Tris buffer/TFE = 1:1 (v/v) was determined from the molar ellipticity at 222 nm using the equation of Chen et al. [23].
The minimal inhibition concentration (MIC) of bacterial growth and the half maximal concentration (EC_{50}) of peptide induced haemolysis were determined as described.

reduce selectivity. The most selective magainin peptide seems to be M2a itself, however, the overall antibacterial activity is poor.

4. Discussion

Our investigations reveal that hydrophobicity, the hydrophobic moment and the angle subtended by the positively charged helix face are effective modulators of antibacterial and haemolytic activity and useful variables to modify membrane selectivity of cationic, potentially helical, membrane active peptides.

Although there is consensus that membrane active peptides exert their activity in a two-step process by (i) binding and (ii) disturbance of the lipid matrix, the mechanism of antibiotic and cell lytic activity is a matter of debate. The absence of detailed structural information for the peptides at the site of action and the complexity of the structure of biological membranes seriously hamper the interpretation of biological data.

The membrane of erythrocytes is composed mainly of zwitterionic phosphatidylcholine and phosphatidylethanolamine with few acidic lipids in the outer leaflet [27]. Thus, electrostatic peptide membrane interactions should be of reduced importance and the pronounced differences in the haemolytic activity of KLA and magainin peptides cannot be explained by the difference is the net positive peptide charges. Hydrophobic peptide–membrane interactions should determine the haemolytic effect. The direct correlation between haemolytic activity and peptide hydrophobicity as well as the hydrophobic moment supports a direct peptide interaction with the electrically neutral lipid matrix of the blood cells and reflects a change in affinity and/or a modified ability to insert into the membrane. A similar correlation between peptide hydrophobicity and the hydrophobic moment, on the one hand, and haemolytic activity has been observed for PGLa (another member of the magainin peptide family) and several model peptides [28,18].

The peptide concentrations necessary to completely inhibit bacterial growth are often much lower than the EC_{50} values for erythrocyte lysis. But interestingly, activity changes induced in the Gram-positive bacteria S. aureus, mostly parallel haemolytic activity leading to the suggestion that hydrophobic peptide–membrane interactions play a decisive role. However, in contrast to red blood cells, Gram-positive bacterial membranes contain negatively charged compounds such as teichoic and teichuroic acids and the carboxyl groups of amino acids found in the peptidoglycan layer [29]. Thus, the low MIC values against S. aureus compared to EC_{50} could be explained in part by the demonstrated increased peptide affinity to negatively charged surfaces [14,15]. The differences in the activity of KLA and magainin peptides can be related to the different cationic charges of the two sets of peptides (+6 and +4, respectively) as found also for other model peptides and antimicrobial peptide analogs [17,5].

Gram-negative bacterial envelops are complex structures composed of an inner membrane which is comparable to mammalian cells [30] and an outer membrane with a high degree of negatively charged lipopolysaccharides located on the exterior surface of the outer membrane. To reach the target, the cationic peptides have to overcome the barrier of the Gram-negative outer membrane. This first step, suggested to be a reversible outer membrane permeabilization, should be

facilitated by the high membrane bound concentration of the cationic peptides. The second step is suggested to be irreversible inner membrane damage leading to cell death [31]. At this membrane, hydrophobic interactions become dominant and although the peptide affinity is low, molecules that overcome the outer membrane may disturb the target membrane most effectively with high peptide hydrophobicity, hydrophobic moment and high angle of the charged domain. A comparison of the ability of the peptides to lyse red blood cells and to inhibit the growth of Gram-negative bacteria reveals that changes in structural properties generally more affect the haemolytic than the antibacterial activity. Obviously, electrostatic interactions play the decisive role for the overall effect on Gram-negative bacteria. The change in the dominance of electrostatic and hydrophobic peptide membrane interactions might also provide an explanation for the prokaryotic specificity of peptides such as magainin while other cationic peptides such as melittin bearing a large hydrophobic sequence region are effective against bacteria as well as mammalian cells.

Alterations of structural features that modify peptide activity against red blood cells but have less influence on the antibacterial activity lead consequently to variations in selectivity. Some selectivity for Gram-negative bacteria is exhibited by peptides of moderate hydrophobicity $(-0.025 > H \geq -0.056)$ which have additionally a low hydrophobic moment and low angle subtended by the charged residues (see KLA2, M2a, 100-M2a). However, in comparison with changes in the selectivity for bacteria found with an increase in the positive peptide charge [5], the hydrophobic parameters seem to play a reduced role for specificity. Additionally, these modifications causing antibacterial specificity often also reduce antibacterial activity as seen with M2a peptides.

The two sets of peptides with modified hydrophobic motifs were designed using α-helical parameters for amino acid residues of Chou and Fasman [26]. Although the TFE/buffer system has been found to be less suitable to describe the membrane bound structure of peptides [15] and the helix propensity of individual amino acid residues in membrane environment can be quite different from that in solution [32] and dependent on hydrophobicity [33] we used the helicity under structure inducing TFE conditions to characterize the peptides by evaluating their potential to assume helix conformation [34]. The determination of the conformation of membrane bound peptides is crucial, especially with peptides exhibiting low affinity to the membrane. In the case of incomplete binding, the CD effects observed in the presence of model membranes always reflect the properties of a mixture of bound and free peptides as documented for KLA and M2a peptides [14,15]. Our results show that the modifications in the sequences had little influence on the helical propensity. While peptide hydrophobicity is independent of helicity, deviations from the ideal α-helix change the theoretical hydrophobic moment and the angle subtended by the cationic helix domain, and peptides with a low hydrophobic moment were found to develop reduced helicity [7]. Thus, the low activity of the most hydrophilic KLA and M2a analogs which exhibit a comparably low helicity (KLA3, 59%; and $L^2R^{11}A^{20}$-M2a, 45%) and the comparably high activity of 140-M2a with 75% helical content might be caused by a change in the actual hydrophobic moment.

In conclusion, our investigations lead to the suggestion that

the haemolytic activity of cationic, potentially helical peptides is substantially modulated by the investigated parameters and increases with hydrophobicity, the hydrophobic moment and the angle of the positively charge helix face. An antibacterial selectivity seems to be reached with peptides of moderate hydrophobicity by reducing the hydrophobic moment and keeping the angle subtended by cationic residues small. Further investigations to qualitatively and quantitatively describe the structure-activity relation of the peptides on the level of model membranes are in progress.

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