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Effect of salt on the interaction of Hal18 with lipid membranes

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Abstract One of the major obstacles in the development of new antimicrobial peptides as novel antibiotics is salt sensitivity. Hal18, an α-helical subunit of Halocidin isolated from *Halocynthia aurantium*, has been previously shown to maintain its antimicrobial activity in high salt conditions. The α-helicity of Hal18 in the presence and absence of salt was demonstrated by circular dichroism spectroscopy, which showed that the peptide was mainly unordered containing β-strands and β-turns. However, in the presence of dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylserine (DMPS) vesicles, Hal18 folded to form α-helices (circa 42 %). Furthermore, the structure was not significantly affected by pH or the presence of metal ions. These data were supported by monolayer results showing Hal18 induced stable surface pressure changes in monolayers composed of DMPC (5 mN m^{-1}) and DMPS (8.5 mN m^{-1}), which again were not effected by the presence of metal ions or pH. It is proposed that the hydrophobic groove within its molecular architecture enables the peptide to form stable associations with lipid membranes. The balance of hydrophobicity along the Hal18 long axis would also support oblique orientation of the peptide at the membrane interface. Hence, this model of membrane interaction would enable the peptide to penetrate deep into the membrane. This concept is supported by lysis data. Overall, it would appear that this peptide is a potential candidate for future AMP design for use in high salt environments.

Keywords Antimicrobial peptide · α-Helical · Hydrophobic groove · Salt resistance · Membrane interactive

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Introduction

Antibiotic resistance can be derived from evolutionary adaptation of bacteria via a number of mechanisms including mutation of chromosomal genes, modification of target molecules, regulation of uptake and/or efflux pumps. Resistance can also be induced by the environment (Gootz 2006), and the overuse of antibiotics has led to the rapid emergence of bacterial resistance to many antimicrobials (Amaral et al. 2007). For example, the multidrug-resistant *Pseudomonas aeroginosa* (MDRPA), a pathogen that is known to colonise the lungs of cystic fibrosis (CF) patients, has become resistant to at least three drugs in the following classes: beta-lactams, carbapenems, aminoglycosides and fluoroquinolones (Obritsch et al. 2005). In this latter case, the increased salinity of the bronchopulmonary fluids compounds the problem in these patients since it is known to further decrease the efficacy of the antibiotics (Park et al. 2004). In addition, it is known that high salt conditions can increase colonisation by bacteria, and resistance to treatment has been reported in a range of respiratory conditions such as in bronchitis. Susceptibility to infection is further compounded in CF patients because antimicrobial peptides (AMPs) such as defensins, which are expressed at the epithelial surfaces of the lung and provide a key defence to infections, become inactive in CF patients because of conditions of high salt concentrations, which lead to

enhanced levels of pulmonary infection (Goldman et al. 1997). Given the role of AMPs in host defence within the lung, these compounds may provide useful treatment for infection in CF patients if inactivation by high salt levels can be overcome. Indeed, in low salt environments AMPs have been shown to be active against a wide range of microbes including those with drug resistance. Although there has been a number of studies on AMPs, there have been few investigations into the effects of salts on efficacy even though salt sensitivity of AMPs has been a major obstacle to their development (Dooring and Gulbins 2009).

Based on these observations, salt-resistant α-helical AMPs may have potential as lead compounds for the development of novel treatments against infection in CF patients (Harris et al. 2009). AMPs have been extensively studied, and research has shown that they all exhibit membrane-interactive properties. Although some AMPs appear to attack intracellular targets, in most cases, direct attack on the microbial membrane itself is the primary killing mechanism mediated by these peptides (Bechinger and Lohner 2006; Sato and Feix 2006). However, despite intensive study, there remains a lack of understanding of the structure/function relationships used by α-AMPs. However, in many cases it is shown that this affinity for membranes is dependent on a positive charge on the peptide interacting with anionic lipids within the membrane. The impact of a high salt environment on these initial electrostatic interactions is likely to be a key factor in environmentally derived resistance.

Halocidins are AMPs, which were originally isolated from the hemocytes of the marine invertebrates *Halocynthia aurantium* and are therefore required to function in a high salt environment (Jang et al. 2002). Halocidin is a heterodimer linked by a disulphide bond between the two α-helical subunits, which consist of 15 (ALLHHGLNCAKGVLA) and 18 (WLNALLHHGLNCAKGVLA) amino acid residues respectively. Halocidin and its subunits Hal15 and Hal18 exhibited high antimicrobial activity against methicillin-resistant *Staphylococcus aureus* (MRSA) as well as MDRPA (Jang et al. 2002). Jang et al. (2003) reported that halocidins derive their cationicity from histidine residues and that this is critical for the AMP to maintain its potent antimicrobial activity in the presence of salt. In the present work, the α-helical conformation of Hal18 was investigated with different lipid systems in the presence of $CaCl_2$, KCl, $MgCl_2$ and NaCl, at varying pHs, to give an insight into the mechanism of membrane interaction and to identify the potential for the peptide to be used for the treatment of infections in environments where there is high ionic strength.

Materials and methods

The synthetic peptide, Hal18 (WLNALLHHGLNCAKG VLA), was synthesised by solid-state synthesis and purified by HPLC to purity greater than 95 %, confirmed by electrospray ionisation mass spectrometry (Servern Biotech, UK). The phospholipids dimyristoylphosphatidylserine (DMPS) and dimyristoyl-phosphatidylcholine (DMPC) were purchased from Avanti Polar Lipids (USA). Buffers and solutions for Circular dichroism (CD) and monolayer experiments were prepared from Milli-Q water with a specific resistance of 18 MΩ-cm. All other reagents were purchased from Sigma (UK).

Primary structure analyses

The primary structure of Hal18 was investigated according to hydrophobic moment plot methodology (Eisenberg et al. 1984). Using an 11-residue window, the Eisenberg's normalised consensus hydrophobicity scale (Eisenberg et al. 1982), based on the assumption of amino acid periodicity of 100°, was used to calculate the mean hydrophobic moment, $\langle \mu_H \rangle$, and mean hydrophobicity, $\langle H_0 \rangle$. Here, the $\langle \mu_H \rangle$ representing amphiphilicity as a result of vector summation in the helical wheel was calculated using 'HMOMENT' (http://bioweb.pasteur.fr/seqanal/interfaces/hmoment.html). Both $\langle \mu_H \rangle$ and $\langle H_0 \rangle$ of the segment having highest $\langle \mu_H \rangle$ were plotted on modified Eisenberg's hydrophobic moment plot diagram by Harris et al. (2000) providing the estimation of putative α-helical peptides with obliquely orientated property.

Calcein vesicle leakage assay

The ability of Hal18 to lyse phospholipid vesicles was studied as previously described by Dennison and Phoenix (2011). Either (7.5 mg) DMPC or DMPS was dissolved in chloroform before solvent evaporation under nitrogen gas and dried in a vacuum desiccator overnight. The lipid film was hydrated using either 5.0 mM HEPES (1 ml) containing 70 mM calcein or 5.0 mM HEPES (1 ml), 70 mM calcein containing 100 mM NaCl. The solution was sonicated for 30 min before three cycles of freeze-thawing. Liposomes were extruded 11 times through a 0.1-μm polycarbonate filter using an Avanti Polar lipids mini-extruder apparatus. Calcein-entrapped vesicles were separated from free calcein by gel filtration using a Sephadex G75 column (Sigma), which was rehydrated overnight in 20 mM HEPES, 150 mM NaCl and 1.0 mM EDTA. The column was eluted with 5 mM HEPES, pH 7.5.

The calcein release assay was performed as previously described by Dennison and Phoenix (2011). The fluorescence intensity of calcein was measured using an FP-6500 spectrofluorometer (JASCO, Tokyo Japan), with an excitation wavelength of 490 nm and an emission wavelength of 520 nm. To measure maximum fluorescence, 20 μl of Triton X-100 was used to dissolve the vesicles. The

percentage of dye leakage was then calculated using the following equation:

$$\text{Percentage leakage} = \left(\frac{F - F_0}{F_{\text{Triton}} - F_0}\right) \times 100 \quad (1)$$

where F_0 is the fluorescence intensity of the lipid vesicles, F is the maximum fluorescence intensity in the presence of Hal18, and F_{Triton} is the intensity after the addition of Triton X-100.

Surface activity of Hal18

Surface activity experiments were performed in a 601 M Langmuir Teflon trough (Biolin Scientific\KSV NIMA, Coventry, UK) equipped with Derlin moveable barriers and a Wilhelmy plate. Maintained at a constant area of 15 cm^2, increasing volumes of Hal18 (2 mM) were injected into either a 10-mM Tris pH 6.4 or a 10-mM Tris pH 7.4 subphase through a sample hole using a Hamilton microsyringe. The adsorption of Hal18 at the air/water interface was monitored by increases in surface pressure for 60 min.

Insertion of Hal18 into lipid monolayers at constant area

The ability of Hal18 to penetrate lipid monolayers at constant area was studied. Monolayers were formed by spreading chloroform solutions of either 5 mM DMPC or 5 mM DMPS onto a clean buffer subphase consisting of either 10 mM Tris at pH 6.4 or 7.4. The chloroform was allowed to evaporate for 10 min before the barriers were closed at a speed setting of 10 cm^2 mm^{-1} until a surface pressure of 30 m Nm^{-1}, which is mimetic of naturally occurring membranes (Demel 1974), was achieved. The lipid monolayer was then maintained at the area corresponding to this pressure throughout the experiment. After equilibration of the monolayer, Hal18 was injected into the subphase to achieve a final concentration of 4 μM. These experiments were repeated in the presence of 100 mM NaCl in the subphase at either pH 6.4 or 7.4. Peptide monolayer interactions were recorded as changes in monolayer surface pressure.

Secondary structure of Hal18

CD spectra were recorded on a J-815 spectropolarimeter (Jasco, UK) at 20 °C as previously described (Greenfield 2006). Far-UV CD spectra were collated for peptide in PBS pH 7.4 and pH 6.4 containing either 100 mM $CaCl_2$, KCl, NaCl or $MgCl_2$ in the presence of lipid was recorded. The phospholipids (5 mg ml^{-1}) DMPS and DMPC were dissolved separately in chloroform and dried under N_2 gas. The lipid film was rehydrated using phosphate-buffered saline (pH 7.5) and sonicated for an hour or until the solution was no longer turbid. Four scans per sample were performed over a wavelength range of 260–180 nm at 0.5-nm intervals, a bandwidth of 1 nm and a scan speed of 100 nm/min. The CD spectra were measured using a 10-mm path-length cell. The percentage helical content was estimated using the CDSSTR method (protein reference set 3) from the DichroWeb server (Whitmore et al. 2010).

Results

Theoretical analysis of Hal18

The hydrophobic moment analysis performed on the Hal18 sequence gave a $\langle \mu_H \rangle = 0.421$ and $\langle H_0 \rangle = 0.18$. When plotted on a Cartesian plane, these mean values lie inside the putative oblique-orientated α-helical region of the hydrophobic moment plot diagram (Harris et al. 2000). Hal18 is therefore a candidate for the formation of an oblique-orientated α-helix at the membrane interface (Dennison et al. 2005).

Calcein leakage analysis

Calcein leakage experiments were undertaken to evaluate the ability of Hal18 to induce leakage of DMPC and DMPS vesicles at pH 6.4 and 7.4. Figure 1 shows at pH 6 there was no statistical difference observed in the percentage of calcein release (circa 50 %) induced by Hal18 in the presence of either DMPC or DMPS vesicles [$T = -0.40$; $p = 0.72$]. Furthermore, there was no detectable pH effect with calcein release at pH 6.4 and 7.4 [$T = -1.369$; $p = 0.26$]. Hal18 is exceptionally salt resistant and in the presence of 100 mM NaCl there is no decrease in lytic activity against DMPC and DMPS membranes at either pH 6.4 [$T = -2.95$; $p = 0.06$] or pH 7.4 [$T = 2.87$; $p = 0.065$].

Surface activity of Hal18

The surface activity of Hal18 was investigated at the air/buffer interface at pH 6.4 and 7. 4. Figure 1a shows that for both pHs the surface activity depends on the peptide concentration. Hal18 displayed a high surface activity at the air/buffer interface at pH 7.4 inducing maximal surface pressure changes of 33.3 ± 0.6 mN m^{-1} with a subphase concentration of 4 μM, which would support previous work (Dennison et al. 2008). At pH 6.4 (Fig. 2a) the surface activity induced a maximal surface pressure change of 33.8 ± 0.4 mN m^{-1} with a subphase concentration of 4 μM indicating that pH has no significant effect

[$T = -4.140$; $p = 0.09$] and implying that any histidine effect must be associated with changes in lipid association rather than surface activity.

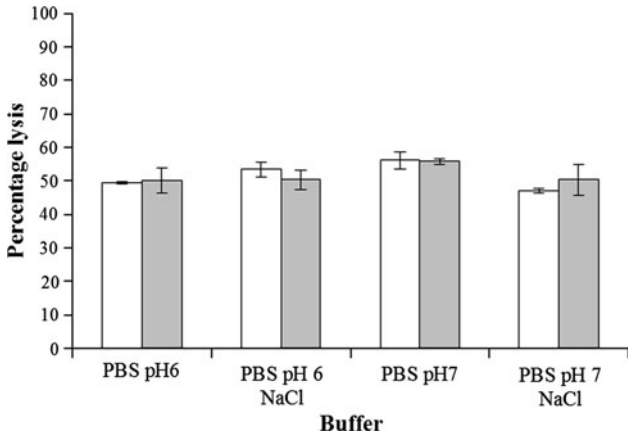

Fig. 1 The percentage calcein leakage of Hal18 in the presence of DMPC (*white*) and DMPS (*grey*) vesicles

Interaction of Hal18 with phospholipid monolayers

Insertion studies using Langmuir monolayers are a widely used technique to study the interactions between peptide and lipid molecules. The ability of Hal18 to penetrate into DMPC and DMPS at constant area was studied using this method (Fig. 2b, c). Figure 2b demonstrates that in the presence of DMPC, at pH 6.4 and 7.4, Hal18 induced pressure increases, which were not statistically different [$T = 1.95$; $p = 0.19$] at circa 5.3 ± 0.15 and 5.0 ± 0.24 mN m^{-1} respectively. Furthermore, Hal18 inserts readily into anionic DMPS (Fig. 2c) membranes at pHs 6.4 and 7.4 inducing maximal surface pressure changes of 8.5 ± 0.15 and 8.4 ± 0.15 mN m^{-1} respectively, which again were not significantly different [$T = 4.33$; $p = 0.05$]. The increased pressure change in the presence of DMPS shows some preference for anionic lipid, which would indicate an electrostatic component to association, but the ability of the peptide to strongly penetrate zwitterionic DMPC would imply that hydrophobic forces also play an

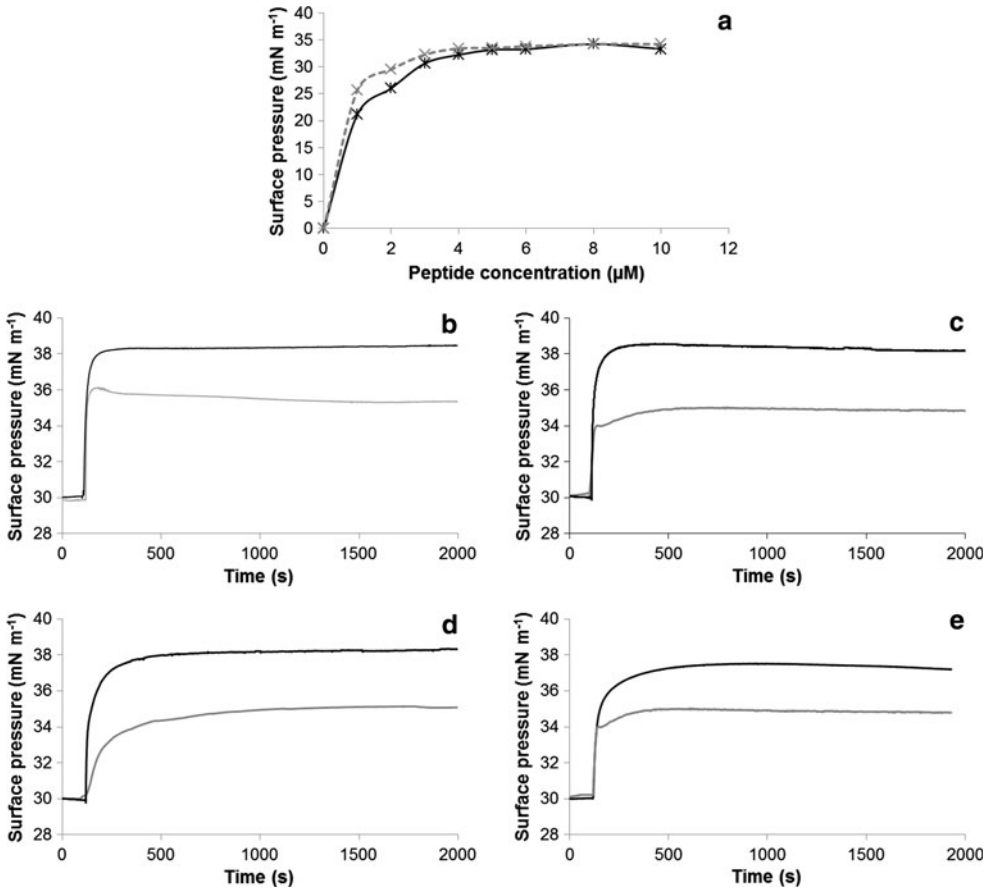

Fig. 2 Monolayer interactions of Hal18. **a** The effect of peptide concentration of surface pressure. Surface pressure values were determined for 30 min after the peptide was injected into the subphase at pH 6.4 (*dotted grey*) and pH 7.4 (*black*). **b** The monolayer interactions of Hal18 into DMPC (*grey*) and DMPS (*black*) monolayers at pH 6.4. **c** Hal18 DMPC (*grey*) and DMPS (*black*) monolayer interactions at pH 7.4. **d** The DMPC (*grey*) and DMPS (*black*) monolayer interactions of Hal18 in the presence of 100 mM NaCl at pH 6.4. **e** The DMPC (*grey*) and DMPS (*black*) monolayer interactions of Hal18 in the presence of 100 mM NaCl at pH 7.4. All these monolayers were repeated five times

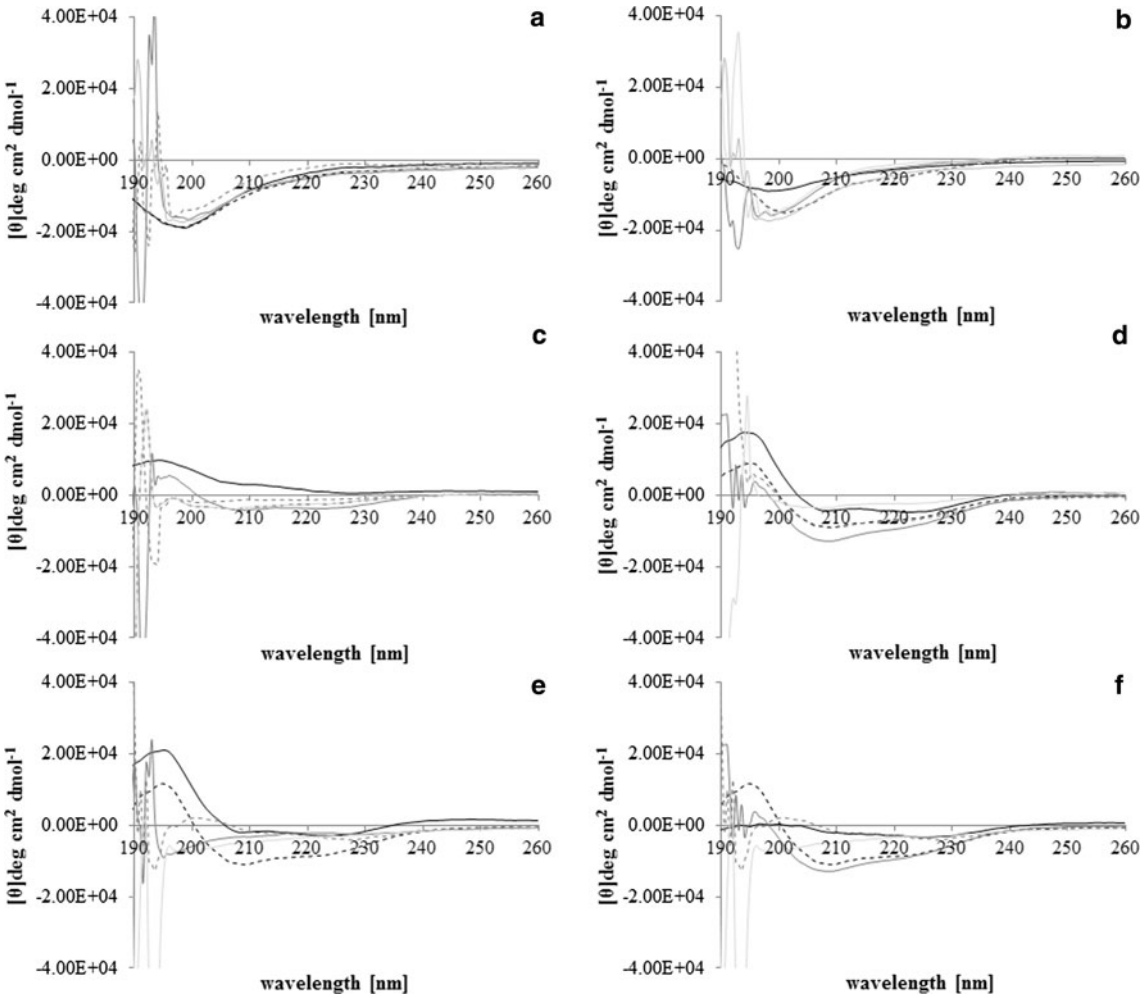

Fig. 3 Solution secondary structure of Hal18. CD spectra of Hal18 in PBS (*solid black*), $CaCl_2$ (*dotted black*), KCl (*solid grey*), $MgCl_2$ (*dotted grey*) and NaCl (*light grey*) at pH 6.4 (**a**), pH 7.4 (**b**), in the presence of DMPC pH 6.4 (**c**), DMPC pH 7.4 (**d**), DMPS pH 6.4 (**e**) and DMPS pH 7.4 (**f**)

important role (Yu et al. 2009). The effect of salt was also investigated and the presence of 100 mM NaCl (Fig. 2d, e) caused no significant decrease in surface pressure change in the presence of either DMPC or DMPS monolayers (4.7 ± 0.20 and 7.2 ± 0.4 mN m^{-1} respectively), which would support the view that the membrane interaction was driven by hydrophobic partitioning rather than electrostatic binding. All these levels of interaction are consistent with disruption of the monolayer acyl chain region by the peptide and are comparable to those reported for other membrane-active peptides (Dennison and Phoenix 2011).

Secondary structure analysis

In the presence and absence of lipid, the effect of $CaCl_2$, KCl, $MgCl_2$ and NaCl on Hal18 structure conformation was investigated at pH 6.4 and 7.4 (Fig. 3). CD spectra obtained at pH 6.4 and pH 7.4 showed that Hal18 in aqueous solution was mainly unordered and contained β-strands and β-turns (Fig. 3a, b). Since many amphiphilic peptides are unordered in solution and require an asymmetric interface, such as that at a membrane surface, to adopt α-helical structure (Dennison and Phoenix 2011), the structural conformation of Hal18 was also examined in the presence of liposomes formed from DMPC or DMPS. In the presence of the zwitterionic model DMPC, at pH 6.4 and pH 7.4 (Fig. 3c, d), the CD spectra demonstrated that Hal18 adopts increased α-helical conformation in the presence of vesicles (42 ± 0.17 %), indicating that the presence of the membrane stabilises the α-helical conformation. The data showed that pH change had no effect on structure. In the presence of metal ions, the helical content remained similar (42 ± 0.95 %, Fig. 4) and statistical analysis showed that the ions induced no significant difference in structure [$T = -1.14$; $p = 0.316$]. The level of helicity in the presence of DMPS vesicles was comparable to that observed with DMPC and again metal ions caused no significant increase [$T = 1.69$; $p = 0.188$] in helicity

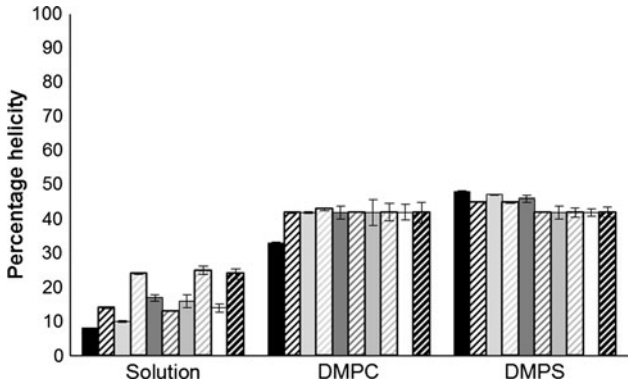

Fig. 4 The percentage of helical structure of Hal18 in the presence PBS (*solid black*), $CaCl_2$ (*light grey*), KCl (*dark grey*), $MgCl_2$ (*grey*) and NaCl (*white*) at pH 6.4 (*solid*) and pH 7.4 (*diagonal lines*)

(42 ± 0.19 %) nor was there a pH effect. However, the CD spectra showed that at both pHs there is a slight decrease in the peptide ellipticity in the presence of metal ions and this may indicate that there is increased peptide aggregation in the presence of DMPC vesicles. There was no significant difference in helical structure between the DMPC and DMPS systems in the presence or absence of metal ions [$F_{19,\ 40} = 0.76$; $p = 0.74$]. The data therefore indicate that the ions do not have any effect on binding and that the peptide can accumulate at similar levels for both a DMPC and DMPS interface.

Discussion

The increased occurrence of bronchial infections in patients with CF has led to investigations to identify salt-insensitive AMPs. Park et al. (2004) demonstrated that in the case of AMPs one of the key factors in salt sensitivity is a reduction in helicity, which in turn would reduce membrane binding and permeablisation leading to a reduction in antimicrobial activity. It has been postulated that the initial stage of AMP activity involves membrane association and only at this point is the amphiphilic helix stabilised (Dennison and Phoenix 2011). Indeed, here we show that in the presence of a lipid environment and metal ions, the helical content of Hal18 remained unchanged, indicating that metal ions had no effect on the peptides ability to associate with a lipid interface (Fig. 4) and adopt an active conformation. These data would imply that the conformational stability of the amphiphilic helical structure at the membrane is an important factor in antimicrobial salt insensitivity. The data show that this salt-resistant Hal18 AMP is not prevented from membrane association and adoption of the α-helical conformation at the membrane interface by salt-shielding effects. This would indicate that whilst many AMPs are dependent on electrostatic forces for membrane association, this salt-resistant peptide is not.

It is well established that histidine residues, which are highly charged only at low pH, are able to promote the interaction of α-helical AMPs with microbial membranes under acidic conditions, for example, as seen with clavanins and clavaspirin from the tunicate *Styela clava* and hebraein from the hard tick *Amblyomma hebraeum* (Lai et al. 2004; Lee et al. 2001; Lehrer et al. 2003). Other researchers have shown that the positive charge acquired by a histidine residue at low pH facilitates the membranolytic antibacterial activity of some defence peptides (Lai et al. 2004), and furthermore, since Hal18 contains two histidine residues these could also serve as potential metal ion binding ligands. The histidine residues have previously been proposed to form the basis for the salt-resistant activity of Hal18 (Lai et al. 2004; Lee et al. 2001; Lehrer et al. 2003). In our investigations in the presence of a lipid environment, metal ions and varying pH, the helical content of Hal18 remained unchanged, indicating that neither metal ions nor pH has any effect on the peptide's ability to adopt active conformation at the lipid interface (Fig. 3). Furthermore, the lytic activity of Hal18 remained constant in the presence of salt against either DMPC or DMPS membranes (Fig. 2). Hence the ability of a peptide to disrupt both zwitterionic and anionic vesicle systems at both pHs would further support the view that binding and efficacy are not initiated by electrostatic binding and implies that histidine does not play an overall role in the membranolytic antibacterial activity of the peptide.

Although there is no evidence of electrostatic interactions being the key in the mechanism of interaction or histidine playing a role, an enhanced level of interaction for Hal18 was observed in the presence of anionic DMPS monolayers (Fig. 2). However, the lack of a pH or salt effect would imply the peptide's lytic activity is driven by the overall architecture rather than being charge dependent. Other researchers have shown that the ability of AMPs to interact with microbial membranes upon not only the target membrane (Nguyen et al. 2011) but also the dependent peptide structural characteristics. Wang et al. (2005) introduced the concept of membrane perturbation potential, which assumes that the ability of the peptide to disrupt membranes is due to the characteristics of the peptide surface. Membrane-active peptide structures including those with oblique orientations have been shown to contain hydrophobic grooves, which are bordered by positive charges with negative charges in the middle of the hydrophilic face. Several studies have shown that such hydrophobic grooves can be key to the antimicrobial activity of some peptides (Dennison et al. 2009). Hal18 exhibits such a hydrophobic groove (Fig. 5), which would enable docking onto anionic lipid membrane in a similar way to that observed for other AMPs such as aurein 2.5 (Dennison et al. 2009). Furthermore, consistent with previous studies

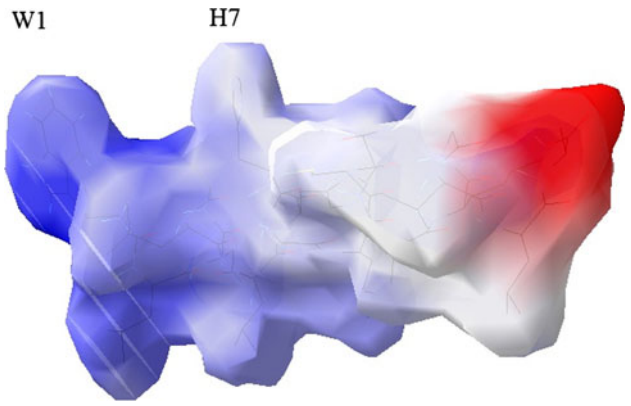

Fig. 5 Electrostatic potential surface of Hal18 modeled using Pymol. The *blue* indicates basic residues, *red*, acidic residues and *white* hydrophobic and neutral residues

(Dennison et al. 2008), Fig. 5 shows that Hal18 has a large hydrophobic surface, which would fit with membrane association, which is enhanced by anionic lipid but where association is driven by hydrophobic partitioning (Dennison et al. 2009).

In previous studies Dennison et al. (2005) have shown that once associated with the membrane lipid, the action of many AMPs involves membrane destabilisation by the use of lipid-interactive oblique-orientated α-helices. Other AMPs such as aurein 2.5, aurein 2.1 and aurein 2.2 possess hydrophobic grooves similar to that shown here (Fig. 5), and previous research has shown that these grooves are not only important for lipid binding, but also drive lipid reorganisation in the bacterial membrane (Dennison et al. 2009). The unfavourable exposure of hydrophobic amino acids to the membrane increases the tilt angle of the peptide, which would be supported by the distribution of hydrophobic residues along the helical long axis (Fig. 5) and fit with adopting an oblique-orientated α-helix as indicated by the $\langle \mu_H \rangle$ $\langle H_0 \rangle$ coordinate pairs. This orientation of the peptide at the membrane interface leads to hydrophobic positive mismatch, where the peptide forces a gap in the hydrophobic region (Hallock et al. 2002). Furthermore, researchers have shown that lysine residues may also assist α-helix orientation by the use of the snorkelling mechanism. According to this snorkelling mechanism, the peptide orientates to enable the flexible hydrophobic sidechains of the lysine residue to extend upwards into the bilayer. As a result, the α-carbon of the lysine amino acid residue engages in electrostatic interactions with the lipid headgroup region enabling the peptide to force a gap in the hydrophobic region leading to negative mismatch and hence a curvature around the peptide in the bilayer. This would support the enhanced levels of monolayer interaction seen for Hal18 in the presence of DMPS.

In summary, Hal18 is highly surface active at salt concentrations greater than physiological salt concentrations. CD and monolayer data show that salt does not inhibit binding nor does it decrease amphiphilic helix content in the presence of either anionic or zwitterionic lipid. Although the peptide shows a preference for anionic lipid, it is not dependent on electrostatic binding and the interaction of Hal18 is probably driven by amphiphilicity via use of the surface groove structure rather than the previous suggestion that it is driven by histidine effect or effect of salt on overall helicity. Furthermore, oblique orientation is seen to be a key factor and aligns with the hydrophobic groove enabling the snorkelling mechanism to lead to hydrophobic mismatch and hence drives membrane disruption, so maximising the disruption of bacterial membrane systems that contain significant levels of anionic lipid.

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