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The effect of pH on the structure, binding and model membrane lysis by cecropin B and analogs

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Abstract

Cecropins are a group of anti-bacterial, cationic peptides that have an amphipathic N-terminal segment, and a largely hydrophobic C-terminal segment and normally form a helix-hinge-helix structure. In this study, the ability of cecropin B (CB) and two analogs to lyse phospholipid bilayers, which have two levels of anionic content, has been examined by dye-leakage measurements over the pH range 2.0–12.0. The two analogs differ from the natural peptide by having either two amphipathic segments (CB1) or two hydrophobic segments (CB3). All these peptides (except CB3 on low anionic content bilayers where it is not active) have maximal lytic activity on both types of bilayers at high pH. However, the pattern of secondary structure formation on these bilayers by the peptides, as measured by circular dichroism (CD), and the pattern of their ability to bind lipid monolayers, as measured using a biosensor, do not directly correlate with the pattern of their lytic ability. CB and CB1 with low anionic content bilayers have secondary structures as measured by CD with a similar pattern to membrane lysis, but binding is maximal near neutral, not high, pH. CB3 has some secondary structures on low anionic content bilayers at low pH and this becomes maximal over the basic range, but CB3 neither binds to nor lyses with these lipid layers. On high anionic content lipid layers, all peptides show high levels of secondary structures over most of the pH range and maximal binding at neutral pH (except for CB3, which does not bind). All three peptides lyse with high anionic content bilayers, but show no activity at neutral pH and reach maximal activity at very high pH. This work shows that pH is a major factor in the capability of antibacterial peptides to lyse with liposomes and that secondary structure and binding ability may not be the main determinants.

Keywords: Membrane; Lysis; Cecropin; Affinity; pH

1. Introduction

Cationic anti-microbial peptides, which form part of the innate immune system, have been identified from many living species and may prove to be of great therapeutic utility as antibiotic resistance spreads [1,2]. Peptides, such as the magainins [3] and the cecropins [4], were found to be effective anti-bacterial agents while having little effect on normal eukaryotic cells [5,6]. They have also been shown

Abbreviations: aBaM, antibacterial/antimalignant; β, fraction of PA (PA/(PA+PC)); CB, cecropin B; CB1, cecropin B-1; CB3, cecropin B-3; CD, circular dichroism; DL, dye leakage; HFP, 1,1,1,3,3,3-hexafluro-2-propanol; PA, phosphatidic acid; PBS, phosphate-buffered saline; PC, phosphatidylcholine; RU, resonance signal units; SPR, surface plasmon resonance

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to be active with various malignant cell lines [5,7] raising the possibility of yet wider therapeutic use for them. They may be thus termed anti-bacterial/anti-malignant (aBaM) peptides [8]. Approaches to using therapeutically these peptides have included topical, intravenous and transgenic applications. They have had varying degrees of success [1]. To make aBaM peptides more efficient as therapeutic agents, a greater understanding of their mode of action and the environments in which they are most effective is required.

In water, helical aBaM peptides do not normally show any secondary structures [9], but will form secondary structures in the presence of polar organic solvents or liposomes [8,9]. NMR studies of cecropin A [10] and sarcotoxin IA [11] have shown that they form helical structures following a helix-hinge-helix motif. The N-terminal helix is amphipathic and the C-terminal helix is largely hydrophobic. Although the mode of action of cationic helical peptides on cell membranes is not known in detail, two main models do exist. One model suggests that the peptides form a helical structure as they approach the membrane. They then aggregate and insert into the membrane, forming pores [12]. Although the membrane-associated peptides may not be the prerequisite for membrane lysis, but it is clear that membrane association is a prerequisite [13]. Many other factors, such as mechanisms of membrane association, lipid composition, etc., are also relevant [14–16]. The other model suggests that the peptides may accumulate on the membrane and destabilize it by a detergent or 'carpet-like' effect [17–19]. Our earlier work has suggested that the characteristics of the peptide might determine the mode of action used [8]. Electrostatic interactions between cationic peptides and anionic lipid bilayers have also been shown to affect the strength of the interaction and the ability of the peptides to cause membrane lysis [20,21].

To investigate the mechanisms of action of aBaM peptides, we have constructed analogs of the naturally occurring peptide cecropin B [4] and investigated their effect on different cell types and liposomes of different compositions [8,9,22]. Cecropin B (CB) is expected to have the helix-hinge-helix structure as determined for cecropin A [10] based on sequence similarity and earlier CD studies, and it also has an N-terminal amphipathic region and a C-terminal hydrophobic region. Two analogs of CB were created to have different physical properties by replacing the N- and C-terminal segments with the other. Thus, CB1 has two amphipathic helices and CB3 has two hydrophobic helices (for sequences, see Section 2.1 below). CB1 achieved enhanced activity on cancer cells [9] and induced different morphological changes in *Klebsiella pneumoniae* and HL-60 leukemia cells when compared with CB [22]. A detailed study of CB, CB1 and CB3 interacting with liposomes of differing anionic content [8] revealed different efficacies of interaction of the peptides with the liposomes as measured by dye-leakage. When liposomes had low anionic content, the amphipathic peptides were more effective at causing dye-leakage. However, as the anionic content of the liposomes increased, the hydrophobic peptide became more effective at causing dye-leakage, even though the strength of the binding interaction between the amphipathic peptides and the liposomes increased [8]. It was also found that the action of the amphipathic peptide involved a two-step kinetic process, while a one-step process was observed for the hydrophobic peptide [8].

In this work, we examine the action of the three peptides (CB, CB1 and CB3) on liposomes with low (15%) and high (75%) anionic content. As the work of Honig, McLaughlin and White [20,21] has demonstrated the importance of the role of electrostatic interactions in the action of cationic peptides on anionic membranes, we consider here the role of pH on the action of the peptides. Over the pH range of 2.0–12.0, the ionization state of the amino acid residues in the peptides and the polar heads of the lipids will change. This will affect both the structure of the peptides and their interaction with the lipids. The secondary structures of the peptides, in the liposome environment, over the pH range is examined by CD measurements and this shows that there are considerable environmental effects. On lipid monolayers with two levels of anionic content, peptide binding is studied by biosensor measurements and liposome lysis is monitored by dye-leakage giving further measures of structural changes. Insights into how the efficacy of aBaM peptides is affected by the range of conditions examined here will help to guide the design of more potent aBaM peptides and pep-

tides that may be more suitable in certain specific conditions.

2. Materials and methods

2.1. Materials

Phosphotidylcholine (PC) and phosphatidic acid (PA) were purchased from Sigma. These products have a purity of 99 and 98%, respectively, and required no further purification. Both PC and PA were used not to construct a mimic cell membrane, such as bacteria, but to model the membranes simply and homogeneously. 1,1,1,3,3,3-hexafluoro-2-propanol (HFP) and Triton X-100 were acquired from Sigma. The fluorescent dye, calcein, was obtained from Boehring Diagnostics. The water used for the experiments was deionized and distilled. The antibacterial peptides, cecropin B (CB), cecropin B1 (CB1) and cecropin B3 (CB3), were synthesized by an Applied Biosystems (ABI) 431 Peptide Synthesizer. The details of the peptide synthesis have been described previously [9]. The sequences of these peptides are shown as below:

CB (NH_2) – KWKVFKKIEK – MGRNIRNGIVKAGP – AIAVLGEAKAL – $CONH_2$

CB1 (NH_2) – KWKVFKKIEK – MGRNIRNGIVKAGP – KWKVFKKIEK – $CONH_2$

CB3 (NH_2) – AIAVLGEAKAL – MGRNIRNGIVKAGP – AIAVLGEAKAL – $CONH_2$

CB1 and CB3 are analogs of CB. CB1 is engineered to have two amphipathic α-helics whiles CB3 has two hydrophobic α-helices (sections underlined). CB itself has both amphipathic and hydrophobic α-helices (sections underlined).

2.2. Preparation of liposomes

The dye-encapsulating (100 mM calcein) and blank vesicles of different compositions ($\beta = 0.15$ and 0.75; $\beta = PA/(PA+PC)$) that were used for CD and dye leakage (DL) measurements were prepared by suspending and then vortexing dried lipids in 10 mM phosphate-buffered saline (PBS) at pH 7.4. The lipid concentration was 10 mM. The suspensions were then sonicated (Laboratory Supplies, model G112 SPIT) for 30 min.e The detailed procedures for the generation of liposomes have been described previously [9]. In the preparation of dye encapsulating liposomes, with $\beta = 0.15$ and 0.75, any free calcein dye and large molecules (or large liposomes) were removed by using a Sephadex G-25 size exclusion column with beads of 1×1000 to 5×1000 (MW) size. The size distribution of the liposomes used in the current experiment is thus relatively homogeneous. The true size distribution of sonicated liposomes will be investigated by using light scattering in future studies. For the pH-dependence experiments using the biosensor, the lipid solutions were prepared at various pH levels (2.0–11.0) before sonication.

2.3. CD measurements

Peptide stock solutions were prepared by dissolving the peptides in a phosphate buffer until their concentrations, as determined by a micro BCA assay (Pierce, USA), were 500 μM. The sample solutions (20 μM peptide with either 20% HFP or 1 mM lipids) used for the experiments were prepared by mixing the peptide stock solution with appropriate amounts of either HFP or vesicle suspensions. The pH of the sample solutions was adjusted by the addition of 1 M HCl or 1 M NaOH. Far UV CD spectra (190–250 nm) of the peptides were measured at 23°C with a Jasco J-720 spectropolarimeter. Water-jacketed quartz cells with a light path of 1 mm were used. The CD data were accumulated over five scans and each experiment was repeated at least two times and the resulting CD curves were averaged and smoothed using the manufacturer's software.

2.4. Dye leakage (DL) measurements

Samples for DL measurements were prepared by adding peptides in 10 mM PBS buffer to suspensions, at varying pH values, of liposomes with encapsulated dyes. The final solution, at each desired pH, had peptide and lipid concentrations of 5 μM and 1 mM, respectively. Peptide-induced dye leakage from liposomes was monitored by the increase in fluorescence. Before the peptides were added, the

solution of dye encapsulating the liposomes, at each pH, was sustained for over 30 min to equilibrate. The fluorescence of the mixed solution was measured with a Perkin-Elmer luminescence spectrofluorimeter (Model LS 50B), with the excitation wavelength set at 496 nm, and the emission was recorded at a wavelength of 517 nm. Both 0 and 100% DL were defined as the fluorescence intensity of a vesicle solution, including the encapsulated dyes, without and with Triton X-100 (0.1%), respectively. The percentage of DL, as a function of pH, induced by the peptides was calculated as follows:

$$\mathrm{DL(pH)(\%)} = [(F(\mathrm{pH}) - F_0(\mathrm{pH}))/ (F_t(\mathrm{pH}) - F_0(\mathrm{pH}))] \times 100 \quad (1)$$

where F(pH) was the fluorescence intensity induced by the peptide, and F_0(pH) and F_t(pH) represented the fluorescence intensities of 0 and 100% DL, respectively, for the given pH. The source of the pep-

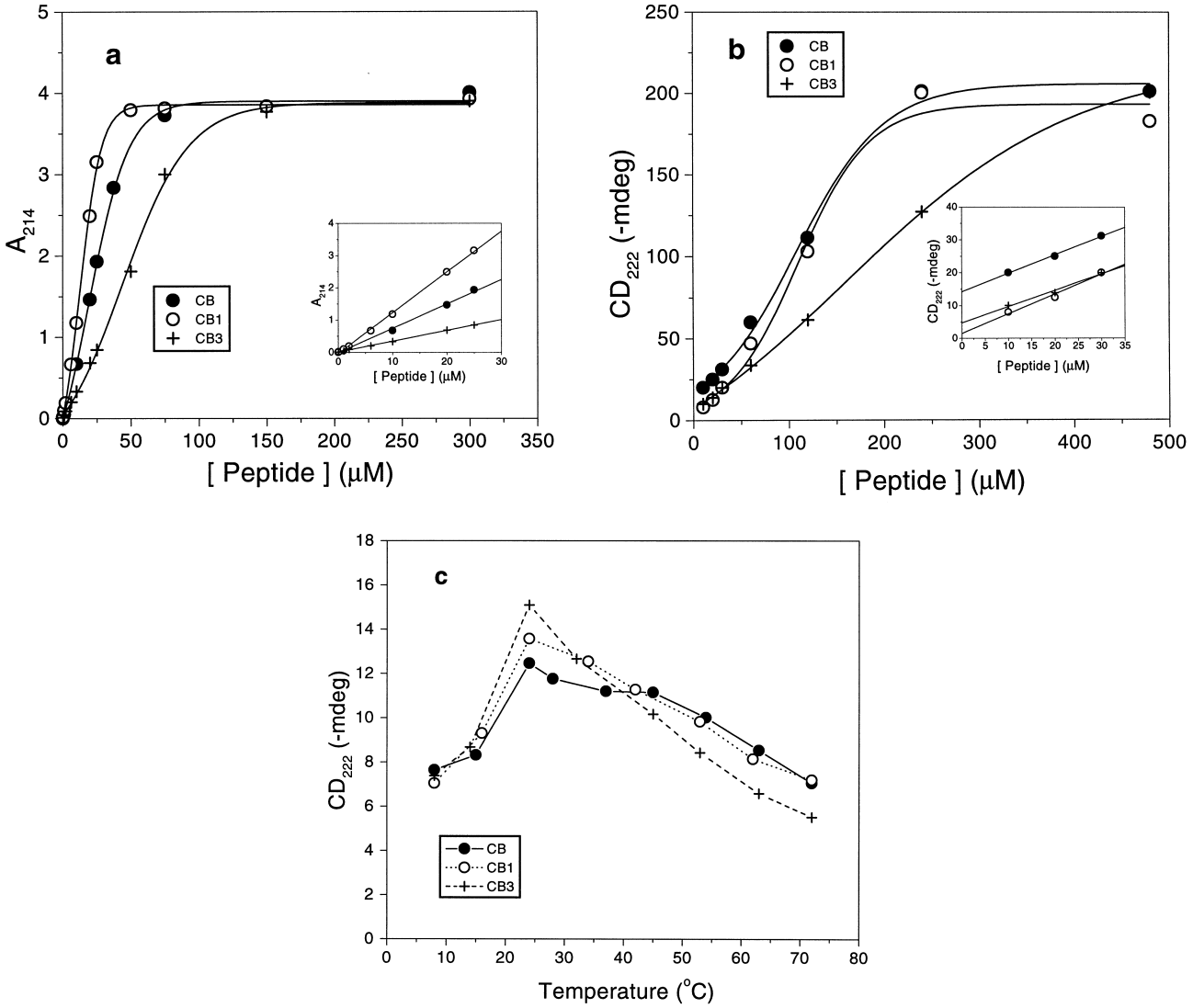


Fig. 1. (a) UV absorption at 214 nm vs. concentration for CB (filled circles), CB1 (open circles) and CB3 (crosses). The inserted panel shows a linear relationship between A_{214} and the concentration of peptide up to at least 30 μM. (b) CD at 222 nm vs. concentration for CB (filled circles), CB1 (open circles) and CB3 (crosses). The inserted panel shows that a linear relationship between CD_{222} and the concentration of peptide up to at least 35 μM. (c) CD at 222 nm vs. temperature (°C) for CB (filled circles), CB1 (open circles) and CB3 (crosses).

tide solution, stirring conditions and the sample volume used for each set of experiments were the same.

2.5. Biosensor binding measurements

A sensor chip, HPA, was coated with liposomes to create a monolayer lipid surface. The coating procedures were as follows. A liposomal solution in 5 mM of Dulbecco's phosphate buffer saline (D-PBS) was run over the sensor chip at a flow rate of 2 μl/min. Irregular and loosely bound adsorbents, such as multiple lipid layers and unbroken liposomes, were removed by increasing the flow rate to 100 μl/min for 5 min and then the surface was washed twice by injecting a solution of 100 mM NaOH at a flow rate of 10 μl/min for 1 min. Surface plasmon resonance (SPR) was used to detect changes in the refractive index of the surface layer as the peptides flowed over and interacted with the lipid coating of the sensor chip. A sensorgram was obtained by plotting the SPR signal against time. A detailed description of measurements by SPR has been given elsewhere [8].

3. Results

3.1. Determination of optimal solution conditions

The aggregation state of the peptides was investigated by the relationship of UV absorption at 214 nm (A_{214}) or CD at 222 nm versus the concentration of peptides in water, as shown in Fig. 1a and b, respectively. Linear relationships are seen in the inserted panels shown in Fig. 1a and b for all three peptides, up to at least 30 and 35 μM, in the A_{214} and CD_{222} measurements, respectively, implying that the peptides do not aggregate within this concentration range. We cannot rule out, however, the possibility that a stable dimer, trimer or even tetramer exists. The detailed investigation of these possible formulas is beyond the scope of this paper. Based on the results, 20 μM was selected as the peptide concentration for the CD experiments. Any variation in the thermal stability of the peptides in 20% HFP was examined by considering the relationship between CD at 222 nm (CD_{222}) and temperature (°C). As shown in Fig. 1c, the extent of α-helix formation, by all three peptides, showed a clear maximum at approximately 23°C, with helical propensity steadily decreasing as the temperature was raised or lowered. Accordingly, the temperature to be used for the current experiments was set at 23°C. The UV absorption and CD measurements were conducted at pH solution values of 7.4 and 6.5, respectively.

3.2. pH-dependence of secondary structures on PA/PC liposomes of $\beta = 0.15$ and $\beta = 0.75$

Typical CD spectra of the peptides at various pH values are shown in Fig. 2. Spectra of CB, CB1 and CB3 with liposomes of $\beta = 0.15$ and $\beta = 0.75$ are in Fig. 2a–c and d–f, respectively. Maximal secondary structure content of $\beta = 0.15$ liposomes was obtained for all peptides between pH 7.0 and 8.0 (Fig. 3a) and it was maintained up to pH 12.0. CB and CB3 show some helical structure at pH 2.0, while CB1 begins to show helical structure only around neutral pH. Below pH 2.0 for CB and pH 6.0 for CB1, the peptides have a random conformation and the solution becomes turbid, which may indicate an aggregation of the interacting liposomes and peptides. As the pH is adjusted back from 2.0 to 7.6 for CB or from 6.0 to 9.0 for CB1, the turbid solutions become clear and the helical structure of the peptides is regained. When the pH is less than 2.0, CB3 is in a random conformation, but the solution remains clear, suggesting that there is no peptide-induced liposomal aggregation. However, the peptide does not regain its helical secondary structure as the pH is raised from 2.0 to 8.0. In $\beta = 0.75$ liposomes (Fig. 3b), CB and CB1 have maximal secondary structures over most of the pH range examined while CB3 shows a sharp increase in its secondary structure up to a pH of about 4.0 and then its secondary structural content steadily increases up to pH 12.0. Below pH 2.5, the peptides are largely in a random conformation and below pH 2.0, all the peptide and liposome solutions are turbid. By adjusting the pH back to 7.4, the turbid solutions become clear, but the peptides do not regain their helical structures.

3.3. pH-dependence of binding affinity to lipid PA/PC monolayers of $\beta = 0.15$ and $\beta = 0.75$

Lipid monolayers at $\beta = 0.15$ and $\beta = 0.75$ were prepared at various pH values (2.0, 4.0, 6.0, 7.4,

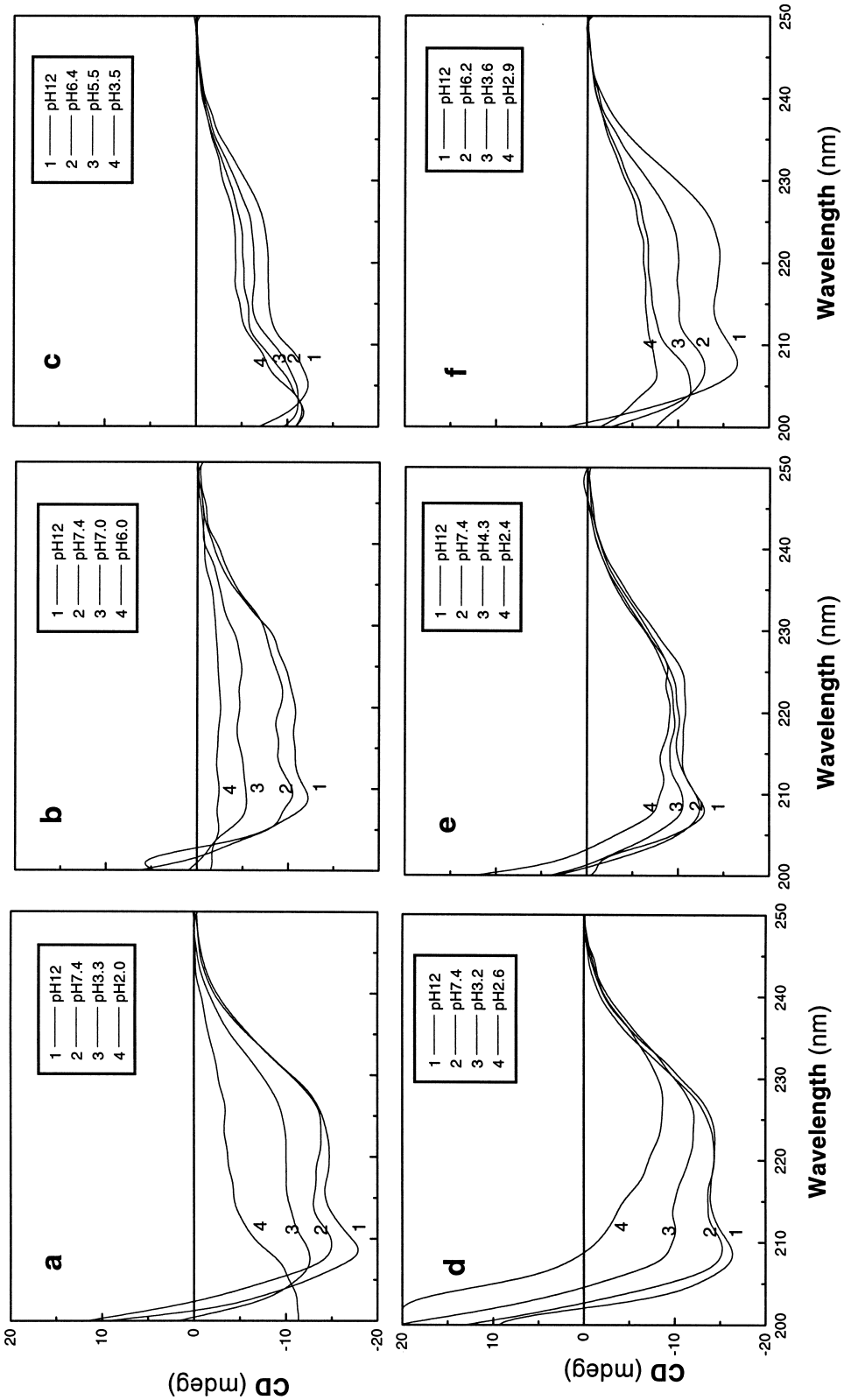


Fig. 2. Typical CD spectra of 20 μM solutions of CB, CB1 and CB3 in the presence of $\beta = 0.15$ and $\beta = 0.75$ liposomes at the pH values indicated in the panels. (a, b and c) CB, CB1 and CB3, respectively, on $\beta = 0.15$ liposomes. (d, e and f) CB, CB1 and CB3, respectively on $\beta = 0.75$ liposomes.

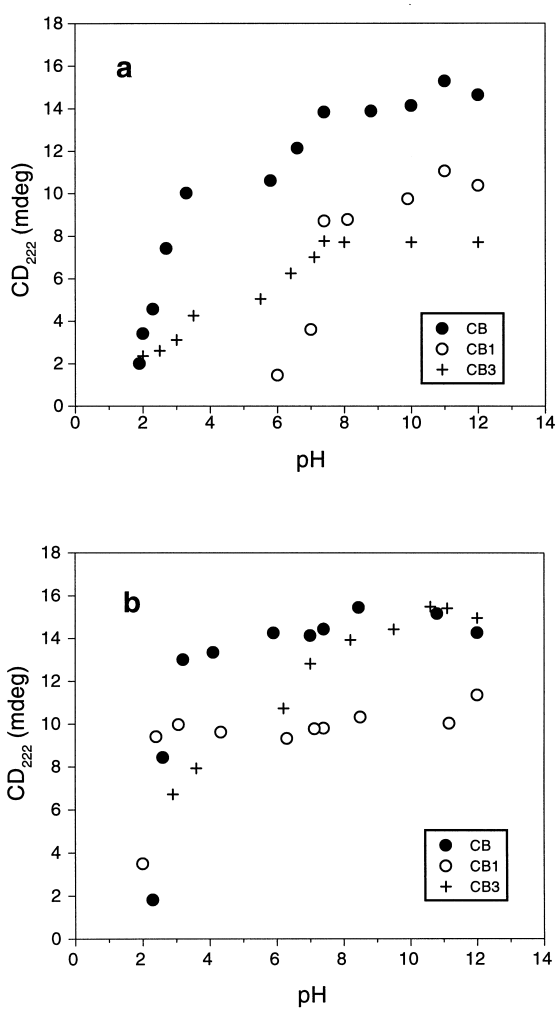


Fig. 3. CD (mdeg) at 222 nm vs. pH for CB (filled circles), CB1 (open circles) and CB3 (crosses) on (a) liposomes of $\beta=0.15$, and (b) liposomes of $\beta=0.75$. This figure includes the data from Fig. 2.

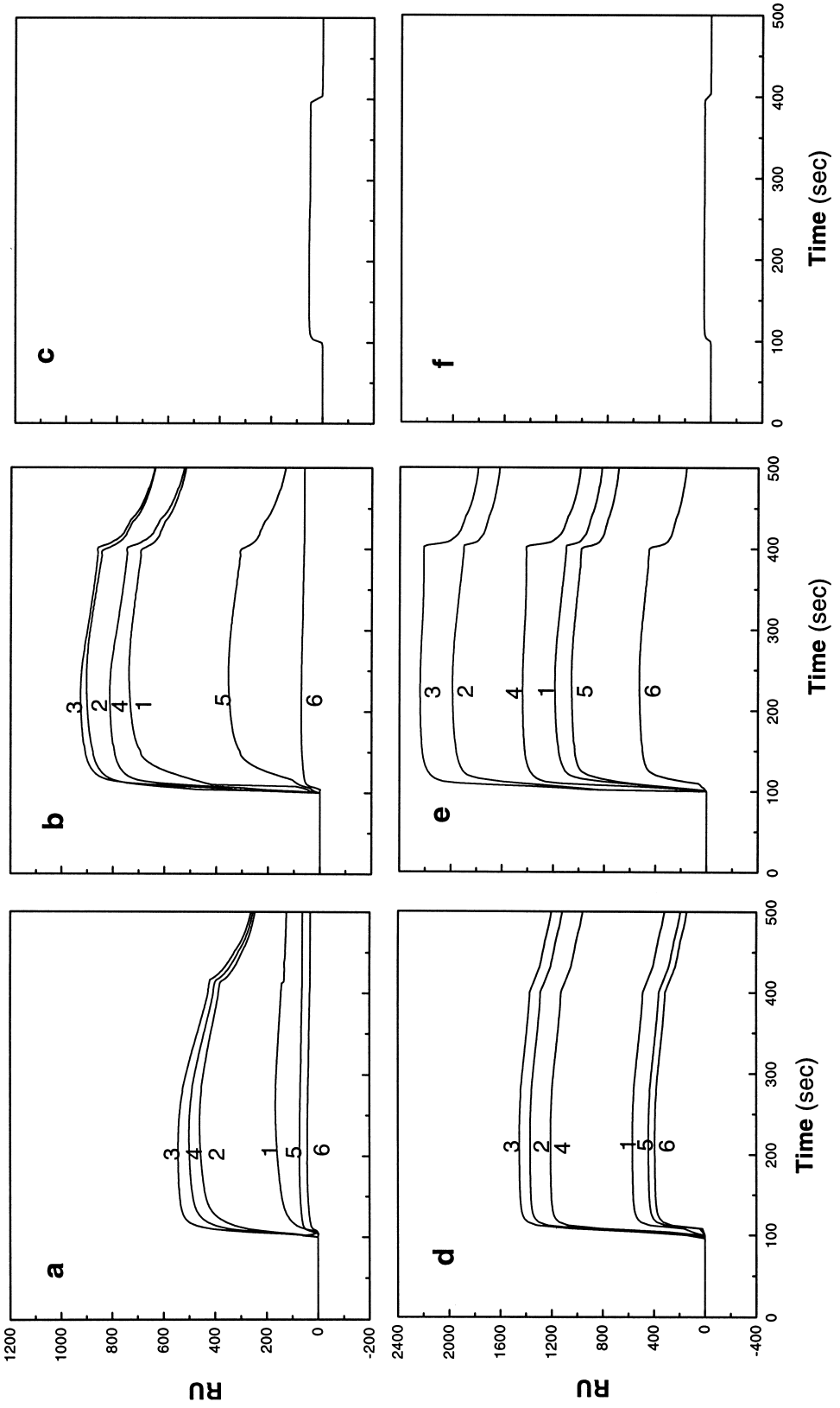
8.0, 11.0) and to coat the HPA sensor chip. A series of solutions of the peptides, with different concentrations (1, 2, 4, 5 and 10 μM) and at the pH of the monolayer, were passed over the monolayer-coated chip. RU levels increased (for CB and CB1) as the peptide concentration increased, indicating that the amount of bound peptide is proportional to its concentration and that the monolayer was not saturated (data not shown). No binding was observed between the monolayers and CB3 at any pH value. Sensorgrams of the binding of the peptides to lipid monolayers of $\beta=0.15$ and $\beta=0.75$ are shown in Fig. 4a–c and d–f, respectively. The peptides were at a concentration of 10 μM and six pH points are shown. Fig. 5a and b shows binding strength as a function of pH from the sensorgrams of Fig. 4, for the peptides interacting with $\beta=0.15$ and $\beta=0.75$ monolayers, respectively. Both CB and CB1 showed stronger binding to liposomes of $\beta=0.75$ and both showed maximum binding capacity to the two monolayers at approximately pH 7.5.

3.4. pH-dependence of dye leakage on liposomes of $\beta=0.15$ and $\beta=0.75$

In this experiment, Triton X-100, a strong membrane lyser, was applied as a reference (DL 100%). The amount of DL gained after the addition of the peptide means that liposomes were lysed by that amount under the assumption that the mode of liposomal lysis is all-or-none (unpublished results; also see [23]). On $\beta=0.15$ liposomes, CB and CB1 caused close to 100% DL above a pH of approximately 8.0, while CB3 did not induce any dye leakage under these conditions (Fig. 6a) and therefore can be considered as a control. CB was able to cause some DL at a pH of 2.0 and this leakage increased steadily to near total leakage at pH 7.0. CB1 had no effect on DL until a pH of about 6.5 and then the extent of DL increased sharply to near complete levels at pH 8.0. For liposomes of $\beta=0.75$, DL was not observed until a pH of 6.0 (Fig. 6b). All peptides showed a sharp increase in DL, with 100% DL being reached at a pH of about 10.0. CB3 is the most effective peptide on the more acidic liposomes, causing substantially more DL in the neutral pH range. CB is able to cause DL at slightly lower pH levels than CB1 can, but CB1 becomes more effective at a pH of about 8.0. The control experiment (open square) was conducted by using liposomes alone.

→

Fig. 4. Surface plasmon resonance sensorgrams of resonance signal vs. time for CB, CB1 and CB3 (10 μM solution) binding to lipid monolayers of $\beta=0.15$ and $\beta=0.75$ at several pH values. (a, b and c) CB, CB1 and CB3, respectively on $\beta=0.15$ liposomes. (d, e and f) CB, CB1 and CB3, respectively on $\beta=0.75$ liposomes. The pH values are (1) 11.0, (2) 8.0, (3) 7.4, (4) 6.0, (5) 4.0 and (6) 2.0. CB3 did not show binding under any conditions. Note that the scales used in a–c are different from those used in d–f.


a
b
c
d
e
f
RU
Time (sec)
1
2
3
4
5
6

A summary of pH-dependence of secondary structures (CD), biosensor binding (RU) and dye-leakage (DL), for CB/CB1/CB3 on liposomes of $\beta = 0.15$ and $\beta = 0.75$ is shown in Table 1. For CB and CB1, the increasing basic nature of the peptide and the increasing acidity of the lipid enhance the interaction between peptide and lipid (see Table 1). For CB3, the peptide did not bind to the monolayer lipids in the biosensor experiments (see Table 1, RU = 0 for both cases of $\beta = 0.15$ and $\beta = 0.75$). However, CB3 was able to form secondary structures (see Table 1, CD for both cases of $\beta = 0.15$ and $\beta = 0.75$). This may be because CB3's association with the lipids is driven by hydrophobic interactions causing it to partition from the solvent to the lipid phase where it forms a helical structure.

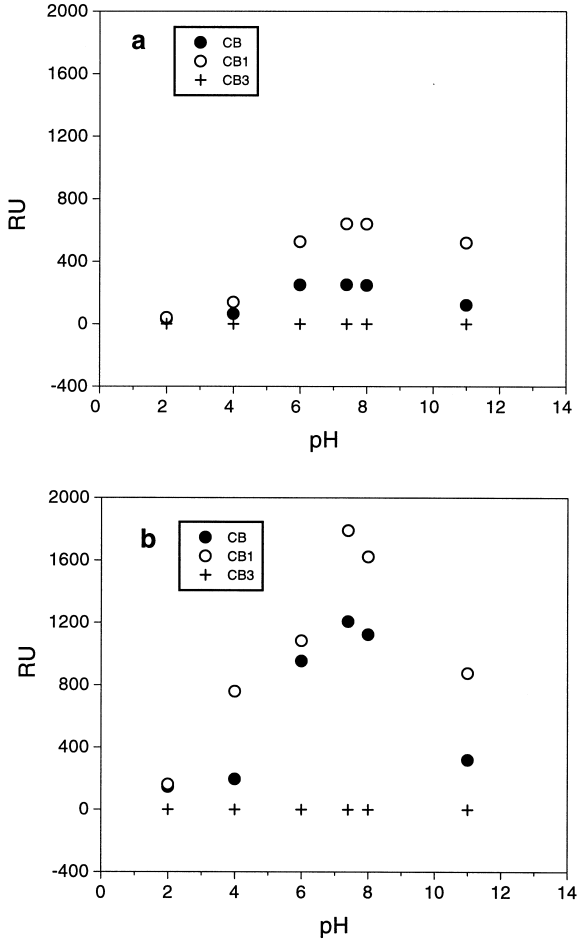


Fig. 5. Binding strength (resonance units) vs. pH for CB (filled circles), CB1 (open circles) and CB3 (crosses) on (a) liposomes of $\beta = 0.15$, and (b) liposomes of $\beta = 0.75$. This figure uses the data from Fig. 4.

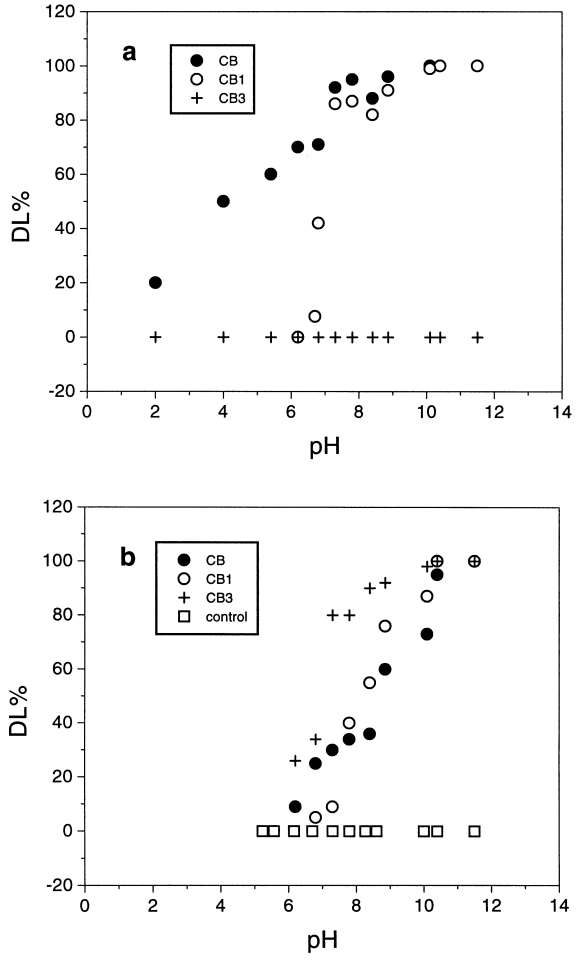


Fig. 6. Percentage of dye leakage (see text for formula) vs. pH for CB (filled circles), CB1 (open circles) and CB3 (crosses) on (a) liposomes of $\beta = 0.15$, and (b) liposomes of $\beta = 0.75$. The control experiments were shown in (a) CB3 (crosses) and (b) liposomes alone (open squares).

4. Discussion

In an earlier work, the action of the peptides CB, CB1 and CB3, at physiological pH, on liposomes with varying degrees of anionic content was investigated [8]. That study showed that there were marked differences in the action of the peptides as the anionic level of the liposome changed. In particular, for the measurements of DL_{50} (the concentration of peptide required to achieve a DL of 50%), both CB and CB1 exhibited a similar pattern with the DL_{50} increasing as β increases. Whereas, CB3 showed the opposite effect, being most efficient at liposome lysis at the highest β level and also having a much smaller range

Table 1
Summary of pH-dependence of secondary structure (CD_{222}), binding (RU) and percentage of lysis (DL%) at selective pHs for CB, CB1 and CB3 on liposomes of $\beta=0.15$ and $\beta=0.75$

	pH	2	4	6	7.4	8	11
$\beta=0.15$							
CB	CD_{222}	3.4	10.3	11.0	13.8	13.9	15.3
	RU	31.0	64.7	249.5	250.0	247.0	120.5
	DL%	20.0	50.0	68.0	92.0	91.0	100.0
CB1	CD_{222}	–[a]	–	1.4	8.7	8.8	11.1
	RU	40.1	138.3	526.1	640.0	639.3	519.2
	DL%	–	–	0	86.0	87.0	100.0
CB3	CD_{222}	2.4	4.4	5.6	7.8	7.7	7.7
	RU	0	0	0	0	0	0
	DL%	0	0	0	0	0	0
$\beta=0.75$							
CB	CD_{222}	1.8	13.3	14.3	14.4	15.0	15.2
	RU	144.6	194.1	953.2	1207.0	1123.0	319.7
	DL%	0	0	9.0	31.0	35.0	100.0
CB1	CD_{222}	3.5	9.3	9.6	9.8	10.1	10.0
	RU	160.5	758.3	1082.8	1790.0	1622.0	875.1
	DL%	0	0	0	9.0	44.0	100.0
CB3	CD_{222}	2.7	8.2	10.7	14.48	14.9	15.4
	RU	0	0	0	0	0	0
	DL%	0	0	24.0	80.0	83.0	100.0

The experimental conditions are described in the text.
[a]– indicates that the data is not available because the solution becomes turbid.

of DL_{50} values than CB and CB1 had. The current work further elaborates on the modes of action of these peptides by examining the effect of pH on the interaction of the peptides with liposomes of low (15%, $\beta=0.15$) and high (75%, $\beta=0.75$) anionic content. We have considered the factors, the ability to form helical secondary structures in the membrane environment and the binding affinity to membranes, that are believed to be central to the lytic action of these peptides [13,24], over the pH range of 2.0–12.0. These data are compared with the lytic activity of the peptides over the same pH range to allow a more precise delineation of the relationship between structure, binding and lytic activity. Indeed, the results obtained from this work can be simply interpreted in terms of the following sequence of chemical equilibria:

$$P_{rc} \overset{K_1}{\leftrightarrow} P_{ss} \overset{K_2}{\leftrightarrow} P_{ss} \ldots \mathrm{LB} \xrightarrow{k_3} (P_{ss}.\mathrm{LB}) \qquad (2)$$

where P_{rc} is the peptide with a random coil, P_{ss} is the peptide with a secondary structure, P_{ss}...LB denote the complex of peptides and lipid bilayers (LB) and (P_{ss}.LB) represents the association of peptide with lipid bilayers. The final stage reflects the product of the lipid bilayer lysis. Both K_1 and K_2 are the equilibrium constants. k_3 is the rate constant. Based on the current experimental observations, the effects of pH on the charge states of the peptides and the liposomes are significant. Therefore, all constants including K_1, K_2 and k_3 are pH-dependent. Both SPR and CD observations can be viewed in terms of the different relative magnitudes of K_1, K_2 and k_3 for all peptides studied. Similarly, the study of dye leakage induced by three peptides can be considered in terms of the effect of pH on the charge and conformational rearrangement of the liposomes. The detailed measurements of K_1, K_2 and k_3 for three peptides used in this experiment may be explored after the detailed investigations of the modes of actions of peptides on lipid bilayers is completed.

Fig. 3 summarizes the helical content of the three peptides in the presence of low (a) and high (b) anionic content liposomes. The general trend is for all the peptides to have a maximal and steady helical content in the basic pH range from approximately pH 8.0–12.0, although CB3 on $\beta=0.75$ liposomes steadily increased its helical content with increasing pH. In low anionic content liposomes (Fig. 3a), in the acidic pH range, CB and CB3 showed some level of secondary structures, which decreased as the pH was lowered, while CB1 showed no evidence of helical content below pH 6.0. It appears that the greater cationic charge of CB1 inhibits the formation of the helical structure and induces aggregation and precipitation in the acidic solvent. The more hydrophobic segments of CB and CB3 should be better able to form a helix in this environment. That CB3, which is the most hydrophobic of the peptides, has a lower helical structure than the intermediately charged CB is probably due to the reduced interaction of this peptide with the liposomes (see Fig. 5 and below). Another influence of the charge of the peptides was seen below the pH at which secondary structures could be detected. Solutions containing CB and CB1 became turbid, suggesting aggregation of liposomes and peptides, but this solution became clear and the secondary structures were restored upon raising the pH. CB3 did not cause the solution to

become turbid, but the loss of helical structure was irreversible. The positive charge of CB and CB1 probably prevented irreversible aggregation of the peptides at low pH.

Regarding membranes of higher anionic content (Fig. 3b), CB and CB1 showed a nearly even level of helical structure over most of the pH range examined. This difference compared to low anionic content liposomes, particularly for CB1, is probably caused by the stronger binding to the more anionic membrane, titrating the positively charged residues and allowing the peptide to be more stable in the acidic solution. The greater CD of CB3 on high anionic content liposomes is consistent with earlier data [8], where it was suggested that the peptide partitions to hydrophobic patches on the membrane surface. At very low pH, all solutions became turbid and, on raising the pH, the solutions became clear, but the helical content was not restored. The higher level of anionic lipids in the solution might cause more lipids to remain bound to the peptides and so inhibit the reformation of the structure seen on the $\beta = 0.15$ liposomes.

Binding to the anionic monolayer lipids was stronger for CB1 than for CB on both types of monolayers as would be expected given the greater cationic charge of CB1 (Fig. 5a,b). The observation that binding to the more anionic monolayer was stronger for both peptides is also consistent with an increase in the electrostatic interaction between peptides and monolayers. Binding by CB3 could not be detected on either type of monolayer, as was seen earlier at physiological pH on lipid monolayers of varying anionic content [8]. In the current work, it can be seen that the maximum binding interaction between the peptide and the lipids occurs around physiological pH and decreases as the pH becomes more alkaline or acidic. At high alkaline pH, the lysine side chains in the peptides will become substantially deprotonated and this would be expected to decrease the strength of the binding. In the acid environment, there would be some protonation of the PA lipid head groups, which would also be expected to weaken the binding interaction. The aggregation seen in the CD studies would not be likely under the flow conditions of the biosensor measurements. These binding and CD studies show that secondary structure and binding are not directly correlated because although for CB and CB1 binding is maximal when helical content is maximal, binding can decrease when helical content does not. In the case of CB3, there is no binding, even though a secondary structure is present. Although the absence of a change in the surface plasmon resonance signal cannot be directly correlated to an absence of binding, the measurements of binding of the three peptides presented here are all relative. The absolute data are not reported. We understand that the surface association of peptides results in high RU changes, such that the change may be beyond detection when the interfacial intercalation occurs. Therefore, we conclude that the absence of large RU signals indicates the absence of binding. Moreover, the difference in RU values observed could also imply different binding mechanisms, or a combination effect. In our case (see Figs. 5 and 6), where RU changes are significant, a qualitative correlation between binding and activity is observed.

The lytic activity of the peptides is summarized in Fig. 6. Only CB on low anionic content liposomes was able to show any activity below a pH of about 6.0. On these liposomes, the activity of CB and CB1 was in broad agreement with the secondary structure data, with maximum lytic activity corresponding to maximum secondary structure. There was no activity for CB3 on $\beta = 0.15$ liposomes at any pH, as seen previously at pH 7.4 [8], even though some secondary structure was present over most of the pH range examined. For these liposomes, the lack of activity of CB3 correlated with its lack of binding. While the binding of CB and CB1 was maximal near maximal lytic activity, the binding strength decreased at high pH while lytic activity remained maximal. For CB, at acidic pH, secondary structure, binding strength and lytic activity all decreased as the pH was lowered. CB1 showed liposome binding at acidic pH, but no secondary structure or lytic activity. However, at neutral to alkaline pH, there was a general correlation among secondary structure, binding and lytic activity.

On high anionic content liposomes, the activity of the peptides was somewhat different to their activity on low anionic content lipids. Binding activity, as measured by the biosensor, showed the same pattern although binding was much stronger. Secondary structure formation and lytic activity, however,

were quite different. A high degree of secondary structure was seen for all peptides over the acidic pH range as well as the alkaline range (Fig. 3b). Despite the presence of secondary structure, almost no lytic activity was detected in the acidic pH range. CB3 was the most active lytic peptide over the pH range, as noted before at pH 7.4 on $\beta = 0.75$ liposomes [8] and CB and CB1 had a similar level of activity, with CB1 being slightly more effective. For CB and CB1, the maximal binding strength corresponded to only 40% DL and maximal lytic activity occurred (at high pH) when binding strength was considerably reduced. Again, CB3 did not bind, but unlike the case on low anionic content liposomes, CB3 was active on the high anionic content liposomes. It was suggested previously that the lytic ability of the more cationic peptides (CB and CB1) might be inhibited by their stronger binding to the membrane holding them on the surface and preventing them from rearranging (perhaps as pores) to lyse the membrane [8]. This may also account for the reduced lytic activity at the pH corresponding to the strongest binding. The absence of lysis at acidic pH, even though a secondary structure and binding are present, may be due to the acidic environment not favoring the rearrangement of the peptides. A stronger lipid–peptide interaction may prevent both the precipitation seen for CB1 and the residual activity seen for CB on low anionic content liposomes. The lytic activity of CB3, even though no binding was detected, was suggested previously to be due to the peptides aggregating in hydrophobic patches of the membrane and destabilizing it by a carpet-like effect [8]. On less anionic liposomes, CB3 was less effective, probably as the larger hydrophobic patches prevented the peptide from accumulating to a critical level. At acidic pH, there would be a significant titration of the charge of the acidic lipids, effectively lowering the anionic content of the liposomes. This may be the reason why lytic activity by CB3 is not detected below pH 6.0.

5. Summary

This work has examined the influence of the two factors – secondary structure in the presence of liposomes and liposome binding – thought to be important for the lytic activity of cecropins [13] as measured by dye leakage from liposomes with two levels of anionic content. It has been shown that there is no clear correlation among these attributes through a study of the pH range 2.0–12.0. While maximal lytic activity occurs when the secondary structure is maximal, the secondary structure can be maximal when lytic activity is not and the reduction in lytic activity does not necessarily correlate with a reduction (in some cases there is no change) in secondary structural content. Binding, as detected by biosensor measurements, is not even necessary for lytic activity by CB3 and, for CB and CB1, maximal binding does not correlate with maximal lytic activity. On high anionic content liposomes, there is a considerable lack of correlation between these two attributes. From these results, it can be seen that, in the action of cationic peptides, secondary structure and liposome binding are not the primary determinants of liposomal lysis. The environment in which a peptide is to act will have a major influence on its efficacy, regardless of its level of structure or binding ability. It should be possible to design more effective antibacterial peptides, targeted to particular environments, based on the insights presented here.CB1 and CB3 are analogs of CB. CB1 is engineered to have two amphipathic α-helics whiles CB3 has two hydrophobic α-helices (sections underlined). CB itself has both amphipathic and hydrophobic α-helices (sections underlined).

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