Circular-dichroism and fluorescence studies on melittin: effects of C-terminal modifications on tetramer formation and binding to phospholipid vesicles

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Studies were performed on a series of melittin analogues with selective alterations to the positively charged amino acid sequence at the C-terminus. Fluorescence studies were undertaken using the sole tryptophan residue in the analogues as an intrinsic fluorescence probe for indications of tetramer formation in free solution, and binding and insertion of the melittins into phospholipid bilayers. Studies were performed under conditions of low-salt buffer with increasing concentrations of phosphate added to

promote self-association of the melittin monomers, and also in the presence of phospholipid vesicles. C.d. studies were also performed under conditions of increasing phosphate concentrations and in the presence of lipid vesicles to monitor the α -helical content of the melittins. It was found that selective replacement of the C-terminal basic amino acids by glutamine has different effects on self-association, α -helix formation and lipid binding of melittin.

INTRODUCTION

Melittin, a small lytic polypeptide from honeybee venom composed of 26 amino acids [1,2], has a profoundly disruptive effect on both artificial and natural membranes [3,4]; its amino acid sequence is shown in Figure 1. The C-terminal region is notable for the sequence of four basic residues, Lys-Arg-Lys-Arg, at positions 21–24.

Melittin has often been studied under a variety of conditions by fluorescence spectroscopy of the intrinsic probe, tryptophan, at position 19 [5]. The technique of c.d., which is sensitive to protein secondary structure, has also frequently been utilized [6–12]. Melittin has been cited as an excellent model for protein-folding studies as it lacks an organized or stable structure in water at submillimolar concentrations [9]. It is proposed that the positively charged C-terminal region has an influence on association of melittin monomers to form tetramers under suitable conditions [13,14]. A recent paper by Hagihara et al. [15] has shown that chemical modification of the amino groups in melittin increases the stability of the tetrameric structure to various extents. The C-terminal charge is also believed to facilitate binding of melittin to phospholipid vesicles [1]. Synthesized melittin fragments lacking the basic C-terminus have greatly reduced lytic activity [16].

In the present study the C-terminus of melittin was selectively modified to alter the charge content in positions 21–24 of the sequence. Tryptophan fluorescence of melittin and its analogues was used to monitor self-association and binding to phospholipid vesicles. The structure properties of the melittin analogues in free solution and in the presence of phospholipid bilayers were also studied by c.d. spectroscopy.

MATERIALS AND METHODS

Synthetic melittin and melittin analogues were obtained from

Peptide Products Ltd and were judged to be more than 95% pure by analytical h.p.l.c. Molecular mass was determined by m.s. and results corresponded to the expected values to within 0.5 Da. The amino acid sequences of the analogues with glutamine replacing arginine at positions 22 and 24 of the sequence (analogue 1), lysine at positions 21 and 23 (analogue 2) or all four positively charged amino acids at positions 21–24 (analogue 3) are shown in Figure 1. The concentrations of melittin stock solutions were determined using a molar absorption coefficient of $5600\text{ M}^{-1}\cdot\text{cm}^{-1}$ at 280 nm [3], using molecular masses of 2790 Da for analogue 1, 2846 Da for analogue 2 and 2790 Da for analogue 3. Phosphatidylcholine (grade 1) from egg yolk was obtained from Lipid Products. Analytical-grade sodium phosphate was purchased from Fisons, and Tris base was from Sigma. All other reagents were of the highest grade commercially available.

Melittin (2846 Da): Gly-Ile-Gly-Ala-Val-Leu-Lys-Val-Leu-Thr-Thr-Gly-Leu-Pro-Ala-Leu-Ile-Ser-Trp-Ile-Lys-Arg-Lys-Arg-Gln-Gln-CONH₂

Analogue 1 (2790 Da): Gly-Ile-Gly-Ala-Val-Leu-Lys-Val-Leu-Thr-Thr-Gly-Leu-Pro-Ala-Leu-Ile-Ser-Trp-Ile-Lys-Gln-Lys-Gln-Gln-CONH₂

Analogue 2 (2846 Da): Gly-Ile-Gly-Ala-Val-Leu-Lys-Val-Leu-Thr-Thr-Gly-Leu-Pro-Ala-Leu-Ile-Ser-Trp-Ile-Gln-Arg-Gln-Arg-Gln-Gln-CONH₂

Analogue 3 (2790 Da): Gly-Ile-Gly-Ala-Val-Leu-Lys-Val-Leu-Thr-Thr-Gly-Leu-Pro-Ala-Leu-Ile-Ser-Trp-Ile-Gln-Gln-Gln-Gln-Gln-CONH₂

Figure 1 Amino acid sequence of melittin and the C-terminally modified melittin analogues used in this study

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C.d. studies

C.d spectra were recorded at room temperature over the range 260–185 nm with a Jasco J600 spectropolarimeter.

Phosphate anion-titration studies on the melittin analogues were performed as follows. The analogues were made up to a concentration of 500 µg/ml in 200 mM sodium phosphate buffer, pH 7.5, and in 10 mM Tris/HCl buffer, pH 7.5. By combining various volumes of these two solutions, a series of solutions of melittin analogues with known phosphate concentrations was produced. The c.d. spectrum of each of the analogues at each phosphate concentration was subsequently recorded in a 0.5 mm-path-length cylindrical silica cell.

Studies were also performed involving titration of phospholipid into melittin and melittin analogue solutions. Large unilamellar egg phosphatidylcholine vesicles were prepared by the extrusion method in 10 mM Tris/HCl buffer, pH 7.5 [18]. The P_i concentration was determined by a modification of the method of Chen et al. [19]. Briefly, 10 µl of sample was heated in acid-washed test tubes for 1 h at 220 °C and then digested with three drops of conc. H_2SO_4 for 30 min. Two drops of 60% (v/v) perchloric acid were then added and the mixture was heated for 1.5 h. The samples were left to cool and 4 ml of a solution containing 0.25% (w/v) ammonium molybdate and 1% (w/v) ascorbic acid in 0.375 M H_2SO_4 was added. The samples were then incubated at 37 °C for 1.5 h and A_{820} was measured against suitable standards.

Melittin analogues were made up to a concentration of 200 µg/ml in 10 mM Tris/HCl buffer, pH 7.5. Known volumes of a 100 mg/ml stock phospholipid solution were then titrated into the melittin solutions. The volumes of phospholipid added were small enough to keep the peptide concentration at between 185 and 200 µg/ml. C.d. spectra were recorded at each titration step in a 0.5 mm-path-length cylindrical silica cell.

Tryptophan emission studies

The emission maximum of tryptophan in the melittin analogues was determined by taking 2 ml of the sample in a silica cuvette, exciting at 280 nm and recording the emission spectrum between 330 nm and 360 nm on a Perkin-Elmer LS 50 luminescence spectrometer at room temperature. Three scans were performed on each sample; the accumulation of the three scans was used to determine the wavelength of the peak of the emission band.

In the case of phosphate anion titration, melittin and melittin analogues were made up to a concentration of 10 µM in 10 mM Tris/HCl buffer, pH 7.5, and in 400 mM sodium phosphate solution, pH 7.5. The volumes of the two solutions were combined to give the desired final concentration of phosphate. For each addition of phosphate, the emission spectrum of tryptophan in each melittin analogue was recorded. The experiment was also performed on solutions of melittin and analogues in 400 mM phosphate, pH 7.5. These spectra were recorded immediately after the solutions had been made up as they tend to be unstable. The data were analysed as described by Schwarz and Beschiashvili [20], by calculating $x = 4[T]/c_p$ (i.e. the fraction of the peptide in the tetrameric state) from the emission maximum where

$$x = (\lambda_M - \lambda) / (\lambda_M - \lambda_T) \quad (1)$$

and λ_M and λ_T are the emission maxima of the monomer and tetramer respectively. If K_a is the association constant for the monomer–tetramer reaction, then [20]

$$x/(1-x)^4 = 4K_a c_p^3 = 8(c_p/c_p^*)^3 \quad (2)$$

where c_p is the total concentration of peptide and c_p^* is the concentration of peptide at which 50% is in the aggregated state.

Figure 2 Shift in emission wavelength maximum of tryptophan for melittin and melittin analogues on addition of increasing concentrations of sodium phosphate buffer, pH 7.5

Excitation was at 280 nm and emission was scanned between 330 and 360 nm. ■, 10 µM melittin; ●, 10 µM analogue 1; ×, 10 µM analogue 2; +, 10 µM analogue 3.

Figure 3 Double logarithmic plots of x , the degree of self-association at equilibrium, for a concentration of 400 mM sodium phosphate buffer, pH 7.5

A straight line with a slope of 3 corresponds to a tetramerization process. ■, Melittin; ●, analogue 1; ×, analogue 2; +, analogue 3.

In the case of phospholipid titration, extruded egg phosphatidylcholine liposomes, prepared exactly as outlined for the c.d. studies, were added to the melittin analogues (5 µM in 10 mM Tris/HCl buffer, pH 7.5). After the addition of each portion of phospholipid, the emission spectrum of the tryptophan residue in the melittin solution was recorded. Melittin binding to phospholipid vesicles has previously been analysed by Schwarz and Beschiashvili [21]. They showed that the binding is in good accord with a model in which the water/membrane partition coefficient (Γ) is modulated by increasing surface charge on the lipid vesicle as the peptide binds. According to this model,

$$r = (\Gamma/\alpha)c_F \quad (3)$$

where $r = c_B/c_L$ and c_B , c_F and c_L are the concentrations of bound peptide, free peptide and lipid respectively. α is given by

$$\ln \alpha = 2v \sin h^{-1}(vbr) \quad (4)$$

where v is the effective charge of the peptide at the interface and b is a dimensionless constant which is determined by geometric factors and ionic strength.

r was determined from the emission maximum, λ , measured for each lipid concentration, by calculating f , the fraction of peptide bound to lipid:

$$f = (\lambda_F - \lambda) / (\lambda_F - \lambda_B) \quad (5)$$

where λ_F and λ_B are the emission maxima of the free and the lipid-bound peptide respectively.

RESULTS

As shown in Figure 2, melittin and its analogues all exhibit a shift of the tryptophan emission maximum to shorter wavelengths with increasing phosphate concentration. The form of the

Table 1 Parameters for peptide self-association in 400 mM sodium phosphate, pH 7.5, and for lipid association in 10 mM Tris/HCl, pH 7.5

n is slope of $\log[\chi/(1-\chi)^4]$ against $\log c_p$; other parameters are defined in the Materials and methods section. Results are means $\pm$ S.D. obtained from curve fitting.

	n	c_p^* (μ M)	$10^{-5} \times \Gamma$ (M^{-1})	ν	λ_B (nm)
Melittin	2.8 ± 0.3	5.0 ± 0.8	2.5 ± 1.4	2.1 ± 0.2	339.4 ± 0.1
Analogue 1	2.5 ± 0.3	6.1 ± 0.9	0.7 ± 0.4	2.4 ± 0.2	341.5 ± 0.1
Analogue 2	3.0 ± 0.3	1.4 ± 0.3	0.8 ± 1.4	1.7 ± 0.7	337.7 ± 0.6
Analogue 3	2.7 ± 0.3	3.1 ± 0.5	0.6 ± 0.25	0.5 ± 0.1	342.7 ± 0.1

Figure 6 Example of the c.d. spectra of melittin analogue 3 on titration with 0, 10, 20, 50, 100 and 200 mM sodium phosphate buffer (pH 7.5)

Peptide concentration was 0.5 mg/ml.

Figure 4 Shift in emission wavelength maximum of tryptophan for melittin and melittin analogues 1, 2 and 3 on addition of egg phosphatidylcholine liposomes

The concentration was 5 μ M peptide in 10 mM Tris/HCl, pH 7.5. Excitation was at 280 nm and emission was scanned between 330 and 360 nm. ■, 5 μ M melittin; ●, 5 μ M analogue 1; ×, 5 μ M analogue 2; +, 5 μ M analogue 3.

Figure 5 Data from Figure 4 replotted as binding isotherms for melittin and analogue 3

■, Melittin; +, analogue 3.

Figure 7 Example of c.d. spectra of melittin analogue 1 in 10 mM Tris/HCl, pH 7.5, with 0, 0.3, 0.6, 0.9 and 1.2 mM egg phosphatidylcholine liposomes added

Peptide concentration was 0.2 mg/ml.

titration curve varies between the different analogues but in 1M phosphate solution they all reach a similar emission maximum.

The position of the emission maximum was also determined as a function of peptide concentration in 400 mM phosphate and analysed according to eqs. (1) and (2). In calculating x from eqn. (1), the value of λ_M was obtained from the low phosphate region of Figure 2 and λ_T was taken as that measured in 1M phosphate. Figure 3 shows plots of plots of $\log[x/(1-x)^4]$ against $\log c_p$. Values for the slopes of these plots and c_p^* were obtained by linear regression and are given in Table 1. Note that K_a may be calculated from c_p^* by means of eqn. (2).

Figure 4 shows emission maxima of the peptides as a function of lipid concentration. All the peptides exhibit a shift to shorter wavelength with increasing lipid concentration but the maximum shift is different for the different peptides. Values of Γ and v were determined from the data by applying the theory given in the Materials and methods section (see also ref. [21]). The curves in Figure 4 are the best fits by eqns. (3–5) with Γ , v , λ_F , λ_B as

Figure 8 Variation in $\Delta\epsilon$ values of melittin (see ref. [8]) and analogues on addition of various concentrations of sodium phosphate buffer, pH 7.5

Melittin concentration was 1.0 mg/ml and analogue concentrations were 0.5 mg/ml. ■, Melittin; ●, analogue 1; ×, analogue 2; +, analogue 3.

Figure 9 Variation in $\Delta\epsilon$ values of melittin and melittin analogues on addition of increasing concentrations of egg phosphatidylcholine liposomes

Buffer was 10 mM Tris/HCl, pH 7.5. Peptide concentrations were 0.2 mg/ml. ■, Melittin; ●, analogue 1; ×, analogue 2; +, analogue 3.

adjustable parameters. The parameter b was taken to be 39 [21]. Values of Γ , v and λ_B obtained from curve fitting are collected together in Table 1. Figure 5 shows data replotted from Figure 4 in the form of binding isotherms to illustrate further the differences between melittin and analogue 3.

An example of the c.d. spectra of analogue 3 titrated against increasing concentrations of phosphate buffer is shown in Figure 6, and Figure 7 shows a phospholipid titration into analogue 1. The values of $\Delta\epsilon$ ($M^{-1}\cdot cm^{-1}$) at 222 nm are plotted in Figures 8 and 9 for all the analogues for titration of phosphate and phospholipid respectively into the peptide solutions.

DISCUSSION

C.d. and fluorescence studies provide complementary methods of investigating secondary structure, self-association and lipid binding of melittin. The c.d. spectrum of melittin in dilute solutions at low ionic strength is characteristic of an irregular structure under such conditions. Increasing melittin concentration or ionic strength and in particular the addition of bivalent anions results in an increase in negative dichroism in the region 210–230 nm which is characteristic of α -helix formation [13]. In aqueous solution, α -helix formation is associated with a transition from the monomeric to the tetrameric state [17]. C.d. spectroscopy can also be used to investigate melittin binding to lipid membranes, as association with the membrane promotes an α -helical conformation [1,6].

Tryptophan fluorescence is sensitive to the polarity of the environment. The wavelength of maximum fluorescence undergoes a blue shift in non-polar environments. This shift can be used to follow tetramer formation of melittin as the single tryptophan is shielded from the aqueous medium in the largely hydrophobic interior of the tetramer [22]. A blue shift also occurs on binding to lipid membranes where it is thought that the tryptophan side chain becomes partially buried in the hydrophobic interior [5,23,24].

In this study, we have used c.d. and tryptophan fluorescence to examine the effects of modification of the C-terminus of melittin on tetramer formation and lipid binding. Previous studies on C-terminally modified melittins (see the Introduction and references therein) have used chemical modification of the lysines in melittin to reduce the overall charge of the molecule or give it a net negative charge. Such modifications may influence the secondary

structure of the peptide. In the present study the selective replacement of basic amino acids by glutamine was undertaken, because, in natural mutational substitutions in proteins and peptides, glutamine is the neutral polar amino acid that most frequently replaces arginine and lysine without affecting the structure and function of the native protein [25].

Qualitatively, the three analogues investigated show similar characteristics to melittin, namely α -helix formation and a blue shift of tryptophan fluorescence on the addition of phosphate or lipid vesicles. There are, however, informative quantitative differences from native melittin.

Figure 2 shows that modification of the C-terminal arginine and lysine residues has opposite effects. Peptide self-association occurs more readily as phosphate concentration is increased when lysine is replaced by glutamine (analogue 2) and less readily when arginine is replaced by glutamine (analogue 1). It has previously been shown that high ionic strength favours tetramer formation for melittin by reducing electrostatic repulsion between monomers [11]. Bivalent anions are, however, much more effective than univalent anions in promoting tetramer formation. It has been suggested that this is because they cross-link the positively charged arginine residues on adjacent monomers [26]. The present results can thus be understood by postulating that replacement of lysine residues favours self-association by reducing electrostatic repulsion. This can be understood with reference to the helical wheel representation of melittin which has been used to demonstrate the amphipathic nature of the melittin α -helix [2]. Lys-23 is present on the hydrophobic face of the helix and thus may have a particular influence on tetramer formation. In melittin crystals the only direct electrostatic repulsion in the tetramer is between Lys-23 residues of each pair of opposing monomers [22]. In analogue 1, however, this effect is outweighed by the absence of arginines and hence the inability of phosphate to form cross-links. Analogue 3 in which all four basic C-terminal residues are replaced is intermediate between analogues 1 and 2 with respect to phosphate-induced self-association.

In order to check that the analogues do in fact form tetramers, the dependence of the emission maximum on peptide concentration was measured in 400 mM phosphate. The data are plotted as $\log[x/(1-x)^4]$ against $\log c_p$ in Figure 3, which according to eqn. (2) should give a straight line of slope 3 for a monomer-tetramer equilibrium. Table 1 shows that a slope of 3 is obtained within experimental error for melittin and analogues 2 and 3. For analogue 1, however, a smaller slope is observed, suggesting that this peptide may, at least in part, form smaller oligomers. The values for c_p^* in Table 1 confirm that self-association of the peptides in phosphate solutions occurs in the order analogue 2 > analogue 3 > melittin > analogue 1, as deduced from the phosphate titrations.

C.d. spectra of the peptides were also assessed as a function of phosphate concentration (Figure 8). The c.d. spectra of the analogues in phosphate solutions show an isodichroic point at approximately 203 nm in agreement with previous studies with melittin [13,17] (e.g. Figure 6). This is consistent with a two-state equilibrium between irregular and α -helical forms, which in solution have been correlated with monomeric and tetrameric states respectively [27]. All the peptides showed an increase in the intensity of the negative band at 222 nm with increasing phosphate concentration, indicative of α -helix formation. In contrast with the tryptophan fluorescence measurements, however, there was little difference between the peptides except that analogue 3 showed a greater propensity to form α -helix. This is somewhat surprising as α -helix formation and tetramer formation in melittin are generally thought to be closely correlated. Monomeric helical melittin does, however, form in aqueous

solution on the addition of less polar solvents [14]. Moreover, the data of Bello et al. [28] show that as the pH of aqueous solutions is varied, helix formation significantly precedes tetramer formation. The present data suggest that replacement of basic residues at the C-terminus has relatively little effect on helix formation but strongly affects self-association of the helical peptides.

We have also investigated lipid binding of melittin and its analogues. The magnitude of the maximum tryptophan emission shift varies, with a smaller shift observed for analogues 1 and 3 than for analogue 2 and melittin (Table 1). This suggests that modification of the C-terminus can affect the environment experienced by Trp-19 on lipid binding.

The titration curves in Figure 4 were fitted using the theory of Schwarz and Beschiaschvili [21] as described in the Materials and methods section to obtain values for Γ and v . Although the theory gives good fits to the data, these parameters are very sensitive to small changes in λ values. There are thus quite large uncertainties in the values obtained, which is particularly marked in analogue 2 where the data show more scatter. Nevertheless, the value of Γ in Table 1 for melittin is in reasonable agreement with that of $1.5 \times 10^{-5} \text{ M}^{-1}$ obtained previously [21]. We also agree with previous work that, although melittin has six basic groups, the effective charge at the lipid surface is only about +2 at pH 7.5 [21]. For analogue 1, Γ is reduced but v is the same as melittin within experimental error. There is a suggestion that v is reduced for analogue 2, but the large uncertainty obviates a firm conclusion. Analogue 3 clearly has a reduced value of Γ and a very low effective charge compared with melittin. To illustrate further the difference between melittin and analogue 3, we have replotted the data for these peptides in the form of binding isotherms (Figure 5). These clearly show the lower initial slope (determined by Γ) and decreased curvature (determined by v) of analogue 3. Thus, although a cursory examination of the lipid titrations in Figure 5 indicates that analogue 3 binds more strongly than melittin, this effect is due to the decreased electrostatic repulsion of the peptide when bound to the lipid. The partition coefficient for analogue 3 is actually somewhat lower than that of melittin so that for a large lipid:melittin ratio (the usual conditions in lysis experiments), analogue 3 in fact binds less strongly.

Lipid binding of the peptides was also investigated by c.d. (Figure 9). The spectra were independent of position along the optical path length of the sample compartment, indicating that scattering artifacts are minimal. Because of the relatively small number of data points, we have not attempted to apply the analysis employed for the fluorescence experiments. Qualitatively, however, the data are in reasonable agreement. They show that analogue 3 approaches saturation binding to lipid vesicles at lower lipid concentrations than the other peptides. Analogues 2 and 3 appear to have some helical structure before addition of lipid. The shapes of the lipid-titration curves for melittin and analogues 1 and 2 are rather similar, in agreement with the fluorescence data.

The very high affinity of analogue 3 for phospholipids requires explanation. In the c.d. studies, binding appears to be complete at molar lipid:peptide ratio of about 4:1 (the corresponding ratio is higher in the fluorescence experiments because of the lower peptide concentration employed [21]). This seems to be too low to be consistent with the analogue simply binding to the vesicle surface. In previous studies with melittin, such low stoichiometries have been associated with a lipid phase change, in particular the formation of the hexagonal II phase [29]. This has not, however, previously been observed with phosphatidylcholine. An alternative explanation is that the lipid surface

promotes the formation of tetramers similar to those occurring in solution, so that not all of the bound melittin is in contact with the lipid.

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