Determination of the secondary structure of selected melittin analogues with different haemolytic activities

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In earlier studies, we have reported that minor modifications in the amino acid sequence of melittin result in dramatic changes in its biological activity. In the current study, we have investigated the secondary structure of melittin analogues with either increased or decreased haemolytic activity in order to further our understanding of the structural features involved in the binding and/or insertion of peptides into a phospholipid membrane from solution. This was accomplished by analysing the c.d. spectra of the analogues in solutions of various ionic strength and, sep-

arately, in the presence of micelles. These studies permit the assessment of the effect of small sequence modifications (i.e. single amino acid omission or substitution) on the self-association-induced secondary structure of melittin in aqueous solution, as well as its binding affinity to micelles. It was found that amphipathicity, as well as interchain distances and the orientation of hydrophobic residues, were involved in the induction of stabilized structures.

INTRODUCTION

Melittin, the predominant peptide isolated from honey bee (*Apis mellifera*) venom, is a cytolytic peptide consisting of 26 amino acid residues. It exhibits strong amphipathic surface activity, and is known to adopt various conformations and aggregation states in different aqueous solutions (Habermann and Jentsch, 1967). Melittin lacks an organized or stable secondary structure at submillimolar concentrations in water, whereas it becomes predominantly helical and tetrameric at high pH, as well as at high ionic strength and/or high peptide concentration (Talbot et al., 1979; Bello et al., 1982; Terwilliger and Eisenberg, 1982; Goto and Hagihara, 1992). It also adopts an α -helical conformation in the presence of membranes and surfactants (Knoppel et al., 1979; Vogel and Jahnig, 1986; Inagaki et al., 1989). These properties make melittin an excellent model for the study of the assembly properties of soluble protein oligomers. Elucidation of the molecular interactions involved in the assembly of melittin in a soluble tetramer and/or its cytolytic activity is of value in furthering our understanding of the interactive relationships of peptides and protein subdomains.

We have recently reported the relative importance to the haemolytic activity of each of the 26 individual amino acids making up the amino acid sequence of melittin (Blondelle and Houghten, 1991a,b, 1992; Blondelle et al., 1993). These studies illustrate how minor sequence modifications result in dramatic changes in the biological activity of this peptide. These findings prompted us to analyse the secondary structures of various melittin analogues under a variety of experimental conditions in order to understand better the structural features involved in the interaction of peptide molecules with phospholipid membranes. We report here the secondary structure of selected melittin analogues in solution at various ionic strengths, as well as in the presence of phospholipid micelles, in order to elucidate the potential correlation between the hydrophobic moment, tetrameric folding and the haemolytic activity of melittin.

EXPERIMENTAL

Peptide synthesis

The peptides used in this study are shown in Table 1 and were prepared using simultaneous multiple peptide synthesis as described elsewhere (Houghten, 1985). Peptides were purified by preparative reversed-phase h.p.l.c. using a Waters Milliprep 300. The purity and identity of the peptides were confirmed by analytical reversed-phase h.p.l.c. with a Vydac C18 column (ODS; 25 cm $\times$ 4.6 mm) and by time-of-flight mass spectroscopy using a BIOION 20 spectrometer.

Circular dichroism

Spectra were recorded at 22 ± 2 °C on a Jasco spectropolarimeter, model J-720 (Jasco, Easton, MD, U.S.A.). Fractional helicities (f_H) were calculated as reported by Weaver et al. (1989), and the secondary-structure content was estimated by a curve-fitting procedure (Yang et al., 1986). C.d. spectra were the average of three scans. A stock solution of peptide in buffer (5 mM Mops, pH 7), a solution of 5 M NaCl and a solution of 5.6 mM lysophosphatidylcholine (Sigma, St. Louis, MO, U.S.A.) in buffer were prepared separately. The samples were then prepared by mixing the appropriate solutions at a range of defined ratios. Final peptide concentrations were determined by u.v. spectrophotometry using a Hewlett-Packard 8452A diode-array u.v. spectrophotometer (Palo Alto, CA, U.S.A.) and by gravimetry, melittin being an internal reference. The experimental absorption coefficients for the analogues were measured by u.v. absorbance at 280 nm in buffer in the presence or absence of 7.2 M guanidinium chloride and related to the absorption coefficient of melittin determined by Quay and Condie (1983), $\epsilon_{280} = 5570 \text{ M}^{-1} \cdot \text{cm}^{-1}$. The absorption coefficients $\epsilon_{280} = 5570 \text{ M}^{-1} \cdot \text{cm}^{-1}$ and $\epsilon_{280} = 11140 \text{ M}^{-1} \cdot \text{cm}^{-1}$ were derived from these experiments, assuming an experimental error lower than 2%, and used for the peptides containing one or two tryptophan residues respectively. The concentration of the omission analogue omW19 was estimated gravimetrically using melittin as reference.

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Table 1 Peptide sequences and hydrophobic moments of melittin and its analogues

μ is the hydrophobic moment as calculated by Eisenberg et al. (1984) for the α -helical region of melittin [i.e. residues 1–21; reviewed in Dempsey (1990)].

	Peptide sequence	μ
Melittin	GIGAVLKVLTTGLPALISWIKRKRQQ-NH ₂	5.565
omW19	GIGAVLKVLTTGLPALIS IKRKRQQ-NH ₂	3.675
omI20	GIGAVLKVLTTGLPALISW KRKRQQ-NH ₂	3.465
subL9W	GIGAVLKVVTTGLPALISWIKRKRQQ-NH ₂	5.418
subP14W	GIGAVLKVLTTGLWALISWIKRKRQQ-NH ₂	5.187
subL16W	GIGAVLKVLTTGLPAWISWIKRKRQQ-NH ₂	5.418
subK7D	GIGAVLDVLTGLPALISWIKRKRQQ-NH ₂	5.187
subK7I	GIGAVLIVLTGLPALISWIKRKRQQ-NH ₂	3.780

RESULTS

In this study, the effect on the secondary structure of melittin of a single omission (indicated as omW19 or omI20) or substitution of those amino acid residues found in earlier studies to result in either substantial increases or decreases in haemolytic activity relative to melittin (indicated as subL9W, subP14W, subL16W, subK7D and subK7I) was examined using c.d. spectrometry. It should be noted that, whereas omission of a single residue from a peptide in an α -helical conformation is expected to alter its overall amphipathicity, a single substitution of a residue is not expected to modify the relative orientation of the other residues

throughout the helix. The c.d. spectra of melittin and its analogues in 5 mM Mops buffer (pH 7) are shown in Figure 1(a) (the peptide sequences are listed in Table 1); the α -helical content of each peptide is listed in Table 2. The spectra for melittin and all of the analogues examined, with the exception of subP14W ($[\theta]_{222} = -14300$ degrees $\cdot$ cm² $\cdot$ dmol⁻¹), are indicative of predominantly unordered structures.

It has been shown that melittin can be induced into an α -helical conformation by increasing solution ionic strength. The conformational changes found are related to a monomer-to-tetramer transition (Talbot et al., 1979; Goto and Hagihara, 1992). It is generally assumed that salt reduces the electrostatic repulsion between positive charges, and promotes interchain hydrophobic interactions resulting in the formation of an antiparallel tetrameric α -helical structure. The NaCl titration data found here for melittin (Figure 2) agree with those recently reported by Goto and Hagihara (1992). In a 1.4 M NaCl solution, only melittin and the two analogues subP14W and subK7D were found to adopt an α -helical conformation (minima at 208 and 222 nm; Figure 1b). The α -helical content of melittin increases from 10% in salt-free buffer to 66% in 1.6 M NaCl (Figure 2). As expected, owing to its high α -helical content in salt-free buffer, subP14W became highly structured at lower NaCl concentrations (α -helical content increased from 43 to 76%). SubK7D, which showed the most melittin-like behaviour, also revealed an appreciable salt-induced increase in helix content (14 to 66%; Figure 2), although this analogue showed a higher degree of folding than melittin at lower salt concentrations. The increased helical content observed for subK7D, although slight, was reproducible. In contrast with the above analogues, the c.d.

Figure 1 C.d. spectra in buffer (a) and in the presence of NaCl (b)

The spectra in (a) were recorded in 5 mM Mops buffer, pH 7, and those in (b) in 5 mM Mops buffer, pH 7, containing 1.4 M NaCl. The spectra of omI20, subL9W and subK7I have been omitted for clarity in (a). The spectrum of omI20 is similar to that of omW19, and those of subL9W and subK7I are similar to that of subL16W. —, omW19; ·····, omI20; — — —, subL9W; — — — —, subK7I; ·····, subL16W; — — — —, melittin; ·····, subK7D; — — — —, subP14W.

Table 2 Ellipticity and haemolytic activity of melittin analogues

HD_{50} is the haemolytic dose required to lyse 50% of the cells. The HD_{50} ratio is HD_{50} (analogue)/ HD_{50} (melittin) (Blondelle and Houghten, 1991a, 1992; Blondelle et al., 1993). The buffer was 5 mM Mops, pH 7, and the 'salt' was 5 mM Mops buffer containing 1.4 M NaCl (except for K7I, 0.9 M NaCl). The micelles were lysophosphatidylcholine and the ratio R_H (concentration of lysophosphatidyl/concentration of peptide) was equal to 12. f_H is the fractional helicity.

	Melittin	omW19	omI20	subL9W	subP14W	subL16W	subK7D	subK7I
HD_{50} ratio	1.0	124	31	5.4	0.8	3.2	4.1	> 200
f_H buffer	0.10	0.01	0.08	0.17	0.43	0.19	0.16	0.21
f_H salt	0.58	0.03	0.11	0.16	0.75	0.27	0.65	0.24
f_H micelles	0.66	0.32	0.42	0.43	0.82	0.66	0.87	0.59

Figure 2 Dependence of ellipticity at 222 nm of melittin analogues on NaCl concentration in 5 mM Mops buffer

□, Melittin; ■, omW19; △, omI20; ▲, subL9W; ◇, subP14W; ◆, subL16W; ○, subK7D; ●, subK7I.

Figure 3 Binding affinity curves of melittin analogues

$[\theta]_{222}$ values were calculated for R_H ([lysophosphatidylcholine]/[peptide]) varying from 0 to 12. □, Melittin; ■, omW19; △, omI20; ▲, subL9W; ◇, subP14W; ◆, subL16W; ○, subK7D; ●, subK7I.

spectra for analogues omW19, omI20, subL9W, subL16W and subK7I were virtually invariant on the addition of salt (Figures 1b and 2). These five analogues were found in earlier studies to

have less haemolytic activity than melittin [haemolytic dose required to lyse 50 of the cells (HD_{50}) ratio greater than 1; Table 2]. These results suggest that the inability of these analogues to fold into an α -helical conformation may be responsible for their low haemolytic activity.

Similarly, in the presence of micelles, differences in the tendency of these analogues to form α -helical conformations were determined (Figure 3). Thus subP14W and subK7D showed very high helical content. The binding curve and c.d. spectral analysis of subK7D revealed a virtually 100% α -helical structure. This cannot be related to the slight modification in amphipathicity (as indicated by a lower hydrophobic moment than melittin, μ ; Table 1) or its decreased haemolytic activity relative to melittin (Table 2). The replacement of Lys-7 by isoleucine yielded a decrease in hydrophobic moment (Table 1). This correlates with a dramatic loss in biological activity (HD_{50} ratio > 200; Table 2). Although the ellipticity for subK7I is similar to that of melittin, a difference in the overall shape of the spectra was observed for this peptide on binding to micelles compared with melittin. A 19% β -structure was estimated for this analogue compared with 3% for melittin using the secondary-structure algorithm provided with the c.d. instrument based on Yang et al. (1986) reference c.d. spectra. Although this algorithm is based on the c.d. spectra of known secondary structure of proteins, this method can be used for the comparison of the secondary structure of sequence-related peptides. The omission of Trp-19 or Ile-20, as expected, also altered the overall amphipathicity and therefore its hydrophobic moment (Table 1). These two omission analogues also showed the lowest α -helical content on interaction with lysophosphatidylcholine micelles (Figure 3). Analysis of the c.d. spectra for these bound peptides revealed, however, a significant β -structure content (18% and 23% for omW19 and omI20 respectively).

The other two tryptophan-substitution analogues studied, subL9W and subL16W, have the same calculated hydrophobic moment (Table 1) and exhibit similar, although small, decreases in haemolytic activity (Table 2). However, subL9W was found to be less structured than subL16W on interaction with micelles, as well as in the presence of salt. The estimated β -structure content of subL16W was 7% in micelles, whereas the structure of subL9W was 23%.

DISCUSSION

These studies show that modification through single amino acid omission or substitution can dramatically affect the secondary structure, as well as the biological activity of melittin. The omission of a single residue from a peptide sequence affects its overall amphipathicity. Variation in amphipathicity can be correlated with changes in calculated hydrophobic moment

(Eisenberg et al., 1984). The hydrophobic moments of a variety of peptides have been reported to correlate well with their biological activities [e.g. melittin, δ -toxin and analogues (DeGrado et al., 1981; Thiaudière et al., 1991; Cornut et al., 1993)]. In the present study, the decreases found in the hydrophobic moments relative to melittin for the two omission analogues (omW19 and omI20) correlated with their decrease in haemolytic activity. Furthermore, the haemolytic activities of these two analogues correlated well with their lack of folding in the presence of increasing salt concentration or of micelles. Substantially diminished amphipathicity appears to result in decreased or weaker specific side-chain interactions and/or altered protein-packing constraints which, ultimately, are expected to inhibit tetrameric assembly. The lack of segregation between hydrophobic and hydrophilic amino acids is expected to promote non-tetrameric structures.

Partial β -sheet structures are known to be highly stable if these peptides are on a phospholipid surface (Takahashi, 1990). The estimated β -structure found for the two omission analogues in the presence of micelles would therefore result in a direct interaction between phospholipids and the peptides, but would prevent the occurrence of a perturbation by these peptides of lipid bilayers. It should be noted that melittin analogues cannot assume an amphipathic character when they adopt a β -structure, as was also found for previously reported peptides (Epand et al., 1987; Takahashi, 1990; Lee et al., 1993). This, in turn, would be expected to result in a decrease in haemolytic activity. On binding of analogue omI20 to dihexadecyl phosphatidylcholine liposomes, the shape of the spectra was similar to those obtained on binding to micelles (β -structure content estimated at 30%).

In contrast with the omission of a single residue, substitution of a single residue in a peptide sequence can result in either large or small changes in the amphipathicity, this being dependent on the differences between the hydrophobicities of the residues being exchanged. For instance, substitution of a lysine by an isoleucine (analogue subK7I) resulted in poor folding capability in a similar manner to that found on omitting Trp-19 or Ile-20. Such poor folding capability may be due to a loss of membrane-perturbing properties related to partial β -structure, and correlates with a virtual lack of haemolytic activity. Furthermore, the removal of the positive charge at position 7 may be responsible in part for the large decrease in haemolytic activity of this analogue. In contrast, incorporating a negative charge at this position (analogue subK7D) resulted in an increase in helicity relative to melittin, although this analogue had a hydrophobic moment similar to melittin coupled with only a small decrease in haemolytic activity. The increased α -helical content observed for subK7D can be explained by the presence of a negatively charged residue near the N-terminus of this helical region, which is expected to result in an increase in stabilization. If one envisages an antiparallel arrangement of a tetrameric array for melittin and its analogues, then repulsive interactions between the N-terminus and the highly positively charged C-terminal segments would be expected to be diminished for subK7D compared with melittin. Such electrostatic stabilization has been described for the anti-symmetric four-helix-bundle proteins (Robinson and Sligar, 1993).

The two tryptophan substitution analogues, subL9W and subL16W, have a slightly lower hydrophobic moment than melittin, which can be related to the small decrease in haemolytic activity seen for these analogues. These results correlate with the inability of these analogues to adopt an α -helical structure, and ultimately its tetrameric self-assembly, on increasing salt concentration. Similar results, lower haemolytic activity and lower α -helical content than melittin, have been reported by Weaver

et al. (1989) for the double-substitution melittin analogue (subL9W+subW19L). The c.d. spectra for subL9W and subL16W revealed that these analogues exhibited more than 30% β -structure at an NaCl concentration of 1.0 M. Under these conditions, the near-u.v. c.d. spectra of subL9W revealed unexpected conformational restrictions for the indole rings, which remained virtually unchanged on salt addition. We believe these findings can be related to intramolecular tryptophan-tryptophan interactions, which result in stabilized induced conformational arrangements. We believe this, in turn, can be expected to discourage macro-structured self-assemblies.

Substitutions on the hydrophilic face of an assumed α -helix with hydrophobic residues (subP14W) are generally thought to be less deleterious to protein stability (i.e. tetrameric folding) than substitutions with less hydrophobic residues on the hydrophobic face (i.e. subL9W and subL16W). This is supported by the substantial haemolytic activity retained for subP14W, as well as its ability to fold into a tetrameric α -helical conformation. In solvents that promote the adoption of an α -helical conformation in melittin, Pro-14 has been shown to destabilize the central turn of the helix. Replacement of this central proline by a tryptophan (analogue subP14W) was found, as expected, to lead to a more structured peptide than melittin. These results are in agreement with the higher α -helical content in methanol reported for the melittin analogue in which Pro-14 was replaced by an alanine (Dempsey et al., 1991). Moreover, it is noteworthy that if one assumes an α -helical conformation for the subP14W analogue, then both the Trp-14 and Trp-19 residues would be located on the hydrophilic face of the α -helix. This would be expected to promote intramolecular hydrophobic interactions between the two indole rings which could serve as a nucleation centre in the folding of this segment. This is supported by the indication of a highly restricted conformation for the indole rings of subP14W in Mops buffer as estimated by near-u.v. c.d. spectra.

In conclusion, this series of analogues has been shown to be useful in the study of the structure-function relationships of melittin and a number of melittin analogues, both in solution and on interaction with model membrane systems. In particular, these studies provide insight into the effect of both hydrophilic and hydrophobic residues on the stabilization of anti-parallel α -helices in a four-helix-bundle motif. The results presented here also indicate that, in conjunction with amphipathicity, interchain distances and orientation factors between hydrophobic residues in a polypeptide chain are important elements in the induction of rigid stabilized structures through hydrophobic interactions. It would therefore appear that, as reported by others (Shortle et al., 1990; Zhou et al., 1992), the importance of hydrophobic interactions varies considerably with the relative position of the hydrophobic residues within a protein or self-assembled multi-meric peptide array.

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