The effect of counterions on melittin aggregation

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Melittin, a surface-active polypeptide from bee venom, has an overall hydrophobic *N*-terminus, with basic residues clustered at the *C*-terminus. In aqueous solution melittin exists as a mixture of monomer and tetramer, the monomer adopting a predominantly random-coil configuration, whereas the tetramer is rich in α -helix. The tendency of melittin to aggregate is dependent on the counter-anions present in solution, the effect being most marked with phosphate, decreasing in the order $\text{HPO}_4^{2-} > \text{SO}_4^{2-} > \text{ClO}_4^- > \text{Cl}^-$.

Melittin, the major polypeptide component of bee venom, is a 26-residue peptide, noted for its lytic action on cell membranes. The polypeptide has been extensively used as a probe of membrane structure (Pik & Lawford, 1980) and as a model for peptide-induced membrane lysis (DeGrado *et al.*, 1982) and protein–lipid interaction (Verma *et al.*, 1974). In solution melittin consists of a mixture of monomeric and tetrameric forms, the position of equilibrium being influenced by a number of factors, including ionic strength, pH and concentration. In aqueous solution the monomer possesses a low α -helical content, in contrast with the tetrameric form, which possesses a high α -helical content (Lauterwein *et al.*, 1980; Brown *et al.*, 1980; see Fig. 3). The two extremes of the monomer–tetramer equilibrium have been extensively studied, namely total monomer and total tetramer, and it is generally accepted that at high ionic strength, with, e.g., 1.0–2.0 M-NaCl or -KCl, the tetrameric state is favoured. Such conclusions have been based on gel permeation (Talbot *et al.*, 1979), ^1H -n.m.r. spectroscopy (de Bony *et al.*, 1979; Faucon *et al.*, 1979) and fluorescence spectroscopy (Talbot *et al.*, 1979; Bello *et al.*, 1982). Whereas in the above-mentioned studies results were obtained with concentrations greater than 0.1 mM, Faucon *et al.* (1979) observed that at lower concentrations of melittin (20 μM) the complete formation of tetramer did not occur, even in 2 M-NaCl. At low ionic strength the melittin monomer predominates (Lauterwein *et al.*, 1980; Habermann, 1980), although this is to some degree dependent on melittin concentration.

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Under a variety of conditions, melittin located at an interface has been shown to exist in a monomeric form, and the presence of this amphiphilic structure in the membrane is responsible for many of the membrane perturbations induced by the peptide. It has also been suggested that, of the two forms of melittin, it is the monomer that partitions from the aqueous phase to the membrane surface (Mollay & Kreil, 1973; Dawson *et al.*, 1978; Lauterwein *et al.*, 1979; Hider *et al.*, 1983). Therefore factors that influence the equilibrium between the two forms might be expected to influence the action of melittin on membranes. Clearly, in studies where melittin is used as a probe, it is important to ascertain the monomer concentration, particularly since the position of equilibrium between monomer and tetramer is sensitive to the presence of different counterions (Drake & Hider, 1979; Georgiou *et al.*, 1981; Podo *et al.*, 1982).

The present paper describes the influence of phosphate and related anions on the melittin monomer–tetramer equilibrium in aqueous media.

Experimental

Materials

Melittin was isolated from bee (*Apis mellifera*) venom (H. Mack, Illertissen, West Germany) and determined to be phospholipase-free by gel electrophoresis and pH-stat assay, and to be free of *N* $^{\alpha}$ -formyl-melittin by mass spectroscopy.

Reagents used were of analytical grade, the counterions were used as their sodium salts, and buffers were filtered before use (Millipore HA filters, 0.22 μm).

Methods

C.d. measurements were made with a Jasco J40 CS or J500 D Dichrograph. The results are expressed in terms of molar ellipticity based on an average monomer weight of 110; the units are $\text{degrees} \cdot \text{cm}^2 \cdot \text{mol}^{-1}$. Absorption spectra of the same solutions were recorded with a Cary 17 spectrophotometer.

Results and discussion

The dependence of melittin aggregation on phosphate, sulphate, perchlorate and chloride anion concentration at pH 7.4 at 22°C was monitored by c.d. spectroscopy. The phosphate spectra are shown in Fig. 1. The curves exhibit an isodichroic point at 203 nm, consistent with the findings reported by Brown *et al.* (1980) that melittin exists in two major forms, the monomer and the tetramer complex. Identical isodichroic points were obtained for sulphate, the counterion used in X-ray-diffraction studies (Anderson *et al.*, 1980), perchlorate and

Fig. 1. Influence of phosphate concentration on the c.d. spectrum of melittin at pH 7.4

For details see the text. The concentration of melittin was 0.5 mg/ml.

chloride. Fig. 2 shows the critical dependence of the position of equilibrium on anion concentrations. The extreme sensitivity towards the presence of phosphate anions is clear. This effect is not a result of either changes in ionic strength of the medium, different anions having a wide variation in efficacy, or changes in the strength of hydrophobic interaction between the monomers, as only low concentrations of salt are required to alter the position of equilibrium. With HPO_4^{2-} , for instance, melittin (0.1 mM) is approximately 50% tetramer at 10 mM salt concentration. Instead, the phenomenon appears to be associated with a specific melittin-anion interaction. Indeed, related anions, e.g. selenate and arsenate, were found to increase the α -helical content of melittin in a similar manner to that of phosphate, whereas others, such as acetate, did not.

Both ammonium and guanidinium ions are known to associate with oxyanions (Springs & Haake, 1977; Williams, 1979), guanidinium ions being more efficient, by virtue of their superior hydrogen-bond-donating ability. In such complexes phosphate and guanidinium ions can form two hydrogen bonds simultaneously (Cotton *et al.*, 1973), whereas only one can form with ammonium ions. Thus the dominant interaction between melittin and oxyanions is likely to be centred on guanidinium functions.

The melittin tetramer possesses a high α -helical content, two domains of α -helix being separated by a proline hinge in each monomer (Fig. 3). Recent studies on the structure of melittin fragments have shown that the hydrophobic F_1 portion (residues 1–19) in either 2-chloroethanol or a lipid environment is predominantly α -helical, in contrast with the F_2 portion (residues 20–26), which consists of a less-ordered structure under such conditions (Lavaille *et al.*, 1982). These results indicate that the C-terminal helical section is not as easily nucleated

Fig. 2. C.d. ellipticity of melittin (220 nm) as a function of salt concentration at pH 8.0

For details see the text. $\square$, Na_2HPO_4 ; $\triangle$, Na_2SO_4 ; ∇ , NaClO_4 ; $\circ$, NaCl .

Fig. 3. Amino acid sequence of melittin

The extents of the *N*- and *C*-terminal helices as determined by X-ray crystallography (Anderson *et al.*, 1980), n.m.r. spectroscopy (Brown & Wüthrich, 1981) and structural prediction (Dufton & Hider, 1977) with the use of Levitt (1978) parameters are shown.

as is the *N*-terminal structure. This difference is probably due to the unfavourable electrostatic interactions in the basic (21–24)-tetrapeptide, the positive charges being more easily accommodated in a random-coil structure than in an ordered α -helix. This obstacle to helix formation can, in principle, be overcome in the presence of counterions, which would decrease electrostatic repulsion between the basic residues. Clearly, anions with a greater affinity for the basic residues would be predicted to exert a greater influence on this inter-residue interaction. Indeed, studies on the conformation of poly-(L-arginine) in the presence of various anions have shown that the random-coil- α -helix transition is triggered by HPO_4^{2-} , SO_4^{2-} and ClO_4^- , whereas certain univalent anions, e.g. Cl^- , failed to induce such a change (Ichimura *et al.*, 1978). Similar structural changes associated with specific anions have also been observed in proteins; for instance, HPO_4^{2-} and ClO_4^- anions are known to increase the α -helical content of basic proteins such as histones (D'Anna & Isenberg, 1972) and fish protamines (Toniolo *et al.*, 1979; Toniolo, 1980). As the protamines are strongly basic, with two-thirds of the residues being arginine arranged in clusters of two to four residues, a similar mechanism of helix formation probably occurs. The experimental results given in the present paper indicate that the order of efficiency of inducing α -helical structure in melittin is $\text{HPO}_4^{2-} > \text{SO}_4^{2-} > \text{ClO}_4^- > \text{Cl}^-$, precisely the same sequences reported by Ichimura *et al.* (1978) for the influence on helical content of poly-(L-arginine), confirming the idea that electrostatic interactions play a dominant role in the tetramerization of melittin.

In addition to facilitating the formation of a *C*-terminal helix in melittin, the presence of high-

affinity counteranions can, in principle, (1) minimize electrostatic repulsion between the basic monomers, and (2) provide cross-link sites between two arginine residues, Cotton *et al.* (1973, 1974) having demonstrated that two guanidinium groups are capable of simultaneously hydrogen-bonding to a single orthophosphate moiety.

Both phenomena would favour tetramer formation, and would provide an explanation for the observation that melittin in aqueous ethanol is monomeric, adopting an α -helical conformation, and yet is converted into the tetrameric state on addition of phosphate (Hider *et al.*, 1983).

It is apparent that the relatively high-charge-density tetrahedral anions HPO_4^{2-} and SO_4^{2-} are particularly well suited as counterions for arginine side chains. The concentration and ionization state of phosphate anions are important variables in studies involving melittin solutions.

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