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LYTIC ACTIVITY OF MONOMERIC AND OLIGOMERIC MELITTIN

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The haemolytic activities of melittin and melittin tetramer as induced by high phosphate counterion concentration, were monitored. Monomeric melittin was found to be fully lytic, whilst tetrameric melittin lacked such activity. Under conditions where melittin was fully tetrameric attempts were made to covalently cross-link the native tetramer using a series of different chain length bifunctional imido esters. The cross-linked oligomers were fully lytic under conditions where melittin was demonstrated to lack such activity. This finding, together with molecular weight determinations and circular dichroism studies, indicated that the cross-linked melittin was quite different to the native tetramer. The haemolytic activity of melittin-containing solutions was related to the concentration of monomeric melittin. The effect of reduced dielectric constant (ϵ) on the aggregation behaviour of melittin and its derivatives was found to favour monomeric melittin.

Introduction

Melittin, the major polypeptide isolated from bee venom (*Apis mellifera*) is noted primarily for its marked cytolytic activity [1]. Melittin has a direct lytic effect on membranes, but also exhibits a synergistic effect with venom phospholipase A_2 [2–4]. It has been demonstrated that melittin activates endogeneous phospholipase A_2 [5], the activation of such enzymes leading to increased prostaglandin biosynthesis and destruction of the cell [6]. The mechanism of melittin's direct lytic properties are at present not established. Melittin is an amphiphilic molecule, characterised by an overall hydrophobic N-terminal region (1–20) and a hydrophilic C-terminus (21–26). The secondary structure predicted by Dawson et al. [7] and subsequently established by X-ray crystallography [8] and NMR spectroscopy [9,10] possesses two regions of α-helix separated by a proline 'hinge'.

Studies on the modification of amino groups and the tryptophan residue [11] have shown that no simple correlation exists between the surface and haemolytic activities of melittin. This has also been observed for fragments of the melittin sequence [12]. Thus it would appear that melittin does not behave as a simple cationic detergent. Melittin adopts a largely α-helical conformation in detergent and lipid micelles, and in phospholipid membranes, as demonstrated by circular dichroism [13–16], ^{1}H-NMR [10,15,17,18] and Raman spectroscopy [19].

One concept for the mechanism of the lytic action of melittin, which has recently gained some support is that of melittin inducing a 'wedge effect' [7]. It was proposed that melittin monomer initially interacts electrostatically with the charged lipid head groups, then undergoing an ordering of secondary structure from random coil to high α-helical content. The amphiphilic helices so produced by this conformational change could then insert into the hydrophobic region of the membrane, producing a localised 'thinning' of the bilayer. A synthetic peptide possessing both an

amphiphilic α-helix and a basic C-terminal region has also been shown to be cytolytic [20], indicating that the amphiphilic helices in melittin are critically associated with its activity [21].

Although the 'wedge effect' is centred on monomeric melittin, it has been demonstrated that tetrameric melittin also binds to lipid micelles and membranes [17,22,23]. Indeed, Knöppel et al. [24] have reported that a covalently cross-linked tetramer is equipotent to native melittin, thus implying that lysis results from the tetrameric form of melittin. In contrast Terwilliger et al. [25], on the basis of X-ray diffraction studies on melittin, considered it unlikely that the melittin tetramer would be lytic.

This paper reports experiments designed to clarify the situation and to determine which of the two forms of melittin, either monomer or tetramer is responsible for the direct lytic activity.

Materials and Methods

Melittin (*Apis mellifera*) was isolated from bee venom and treated with *p*-bromophenacyl bromide in ammonium acetate (0.1 M, pH 6.0) at 30°C for 15 h, before use in haemolysis experiments. After treatment with *p*-bromophenacyl bromide, phospholipase activity, as measured by the pH-stat was below detection limits.

Dimethylsuberimidate was purchased from Pierce Chemicals, dimethylpimelimidate from Sigma Chemical Co., dimethyladipimate, succinimidate and malonimidate were a gift from Dr. P. Lind, and dimethylglutarimidate was synthesised [26]. Purity was determined by elemental analysis.

The cross-linking of melittin, except where stated, was for 30 min in 1 ml reaction volume with 2 mg melittin ($7 \cdot 10^{-7}$ mol) and 2 mg cross-linking reagent (approx. $7 \cdot 10^{-5}$ mol) in sodium phosphate buffer (0.5 M, pH 8.3).

Circular dichroism measurements were made using a Jasco J40CS. The results are expressed in terms of molar ellipticity based on an average monomer molecular weight of 110, the units are degree $\cdot$ cm^2 $\cdot$ mol^{-1}. Absorption spectra of the same solutions were recorded with a Cary 17 spectrophotometer.

The concentration of melittin was calculated from the experimentally determined extinction coefficient at 280 nm, Tris-HCl (10 mM, pH 7.4). The content of water was determined by elemental analysis (41%), giving a value of 5660 cm^{-1} $\cdot$ M^{-1} which is in good agreement to the previously reported value [27].

Gel permeation chromatography was performed on Sephadex G-50 (fine, 1.5 × 100 cm) equilibrated with either Tris-HCl (20 mM, pH 7.4) or sodium phosphate (500 mM, pH 7.4).

Polyacrylamide gel electrophoresis was performed on a 10–18% acylamide gradient [28]. Protein samples (20 μg) were prepared by incubation at 20°C for 2 h in the following solution: 8 M urea, 5% SDS, 1% 2-mercaptoethanol in Tris-HCl (0.25 M, pH 6.2). Gels were stained in a 1 : 5 : 1 (by vol.) solution of methanol/water/acetic acid containing 0.12% Kenacid blue for 75 min, destained in a 1 : 2 : 17 (by vol.) solution of methanol/acetic acid/water, then shrunk in 1 : 1 (by vol.) aqueous methanol for 30 min and dried under vacuum. Gels were scanned at 600 nm.

The results obtained with outdated and fresh heparinised blood in haemolysis assays were similar. Blood (50 ml) was centrifuged and washed with NaCl (150 mM) buffered with Tris-HCl (20 mM, pH 7.4) three times. The erythrocytes were maintained at 4°C until required. The washed cells were suspended 1 : 1 (by vol.) in either NaCl (150 mM) buffered with Tris-HCl (20 mM, pH 7.4) or sodium phosphate (500 mM, pH 7.4). Incubations were carried out at 37°C, 2.5 ml of the appropriate buffer and 0.5 ml of the erythrocyte suspension were mixed with melittin samples. After incubation the samples were centrifuged for 30 s on a Beckmann Microfuge, 0.1 ml of the supernatant was diluted to 1 ml with buffer and its absorbance monitored at 578 nm.

Results

(a) Preparation and haemolytic activity of suberimidate cross-linked melittin

Melittin was cross-linked with dimethylsuberimidate both under the conditions described by Knöppel et al. [24] and others outlined in Methods. Products formed by the procedure of Knöppel et al. were found to possess slower running times on SDS-polyacrylamide gel electrophoresis

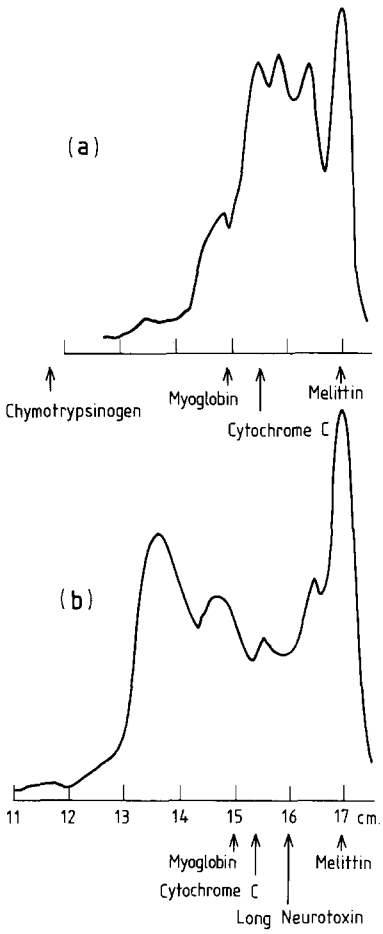

Fig. 1. Comparison of the polyacrylamide gel of melittin cross-linked by dimethylsuberimidate in (a) sodium phosphate (0.5 M, pH 8.3) and (b) triethanolamine-HCl (0.2 M, pH 8.3).

than previously reported (Fig. 1). The major species possessed an apparent molecular weight of approx. 20 000, which is appreciably higher than the native tetramer. The oligomer bands were extremely diffuse, indicating heterogeneity of the suberimidate cross-linked products. Cross-linking with subermidate in the presence of phosphate (0.5 M, pH 8.3), in an attempt to stabilise the tetramer, produced a series of oligomers, apparently with melittin tetramer as one of the products (Fig. 1). Altering the ratio of the cross-linker to melittin (5 : 1, 1 : 1 by weight, respectively) in the phosphate system does not greatly influence the ratio of products formed, all profiles being similar to that depicted in Fig. 1a.

The haemolytic properties of melittin and the suberimidate cross-linked derivatives produced with and without phosphate were identical (Fig. 2), as judged by the criterion adopted by Knöppel et al. [24], in 150 mM NaCl, 20 mM Tris-HCl (pH 7.4). Concentrations are presented in terms of melittin monomer. Performing the experiments at 4°C produced a slight shift, where cross-linked melittin oligomer was more haemolytic than native melittin.

Melittin forms tetramers in phosphate solution (0.5 M) whereas in isotonic saline it is predominantly monomeric [13]. The response of human erythrocytes to melittin in these two solutions was monitored (Fig. 3) and the melittin tetramer was found to be void of lytic activity. The susceptibility of erthrocytes to lysis by Triton X-100 and sodium dodecyl sulphate (SDS) was found to be similar in both media, so that the decrease in lytic activity of melittin in the presence of phosphate was not a consequence of cell shrinkage and concomitant stability towards haemolysis.

The effect of phosphate concentration on erythrocyte lysis (Fig. 4) indicates that the rate of lysis increases with decreasing phosphate concentration, that is as the concentration of monomer increases. In complete contrast the lytic potency of the suberimidate cross-linked melittin

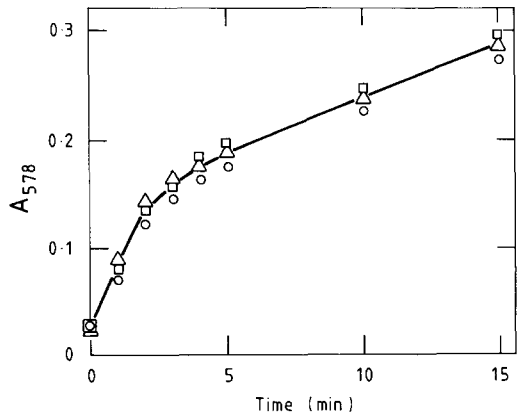

Fig. 2. Induced haemolysis of erythrocytes by melittin (□) in 150 mM NaCl, 20 mM Tris-HCl (pH 7.4); suberimidate cross-linked melittin oligomer (△) in 150 mM NaCl, 20 mM Tris-HCl (pH 7.4) and suberimidate cross-linked melittin oligomer (□) in 500 mM sodium phosphate. Each at 2 $\mu g \cdot ml^{-1}$ of incubation media.

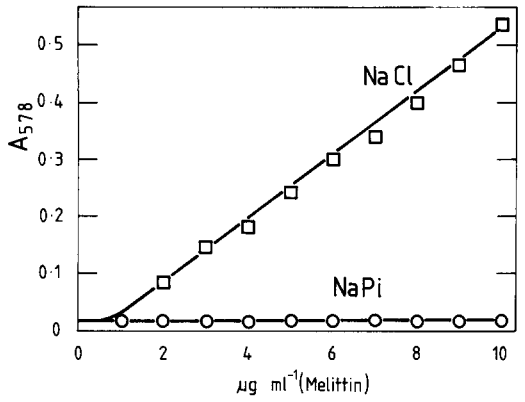

Fig. 3. The effect of phosphate on melittin-induced haemolysis of erythrocytes in 150 mM NaCl, 20 mM Tris-HCl (pH 7.4) (□) and in 500 mM sodium phosphate (pH 7.4) (○).

oligomer, in phosphate buffer, was found to be equal to that of melittin in sodium chloride buffer (Fig. 2).

The results confirm the observation of Knöppel et al. [24] that suberimidate cross-linked melittin is equipotent to native melittin in the lysis of erythrocytes in isotonic saline. Significantly however, the cross-linked oligomer retains its lytic activity in phosphate buffer (0.5 M, pH 7.4), whereas native melittin is void of such activity.

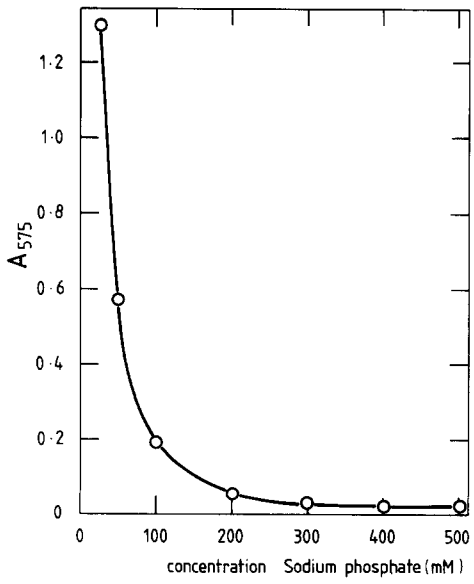

Fig 4. The effect of phosphate concentration on melittin induced erythrocyte lysis, incubation time 1 min.

The polyacrylamide gel profiles (Fig. 1) indicate that some tetramer is present, but the failure to identify a component of molecular weight 12000 on gel permeation (Fig. 6) indicates that a tightly cross-linked product is not formed, and that protein absorption must be occurring.

(b) Cross-linking of melittin with different chain length bifunctional imido esters

In view of the influence of phosphate on the cross-linking pattern of dimethylsuberimidate (Fig. 1), the same system was adopted with different cross-linking reagents in an attempt to isolate the melittin in a covalently locked tetrameric state. The shorter reagents malonimidate, succinimidate, glutarimidate and adipimate give predominantly monomer and dimer, whilst pimelimidate and sub-

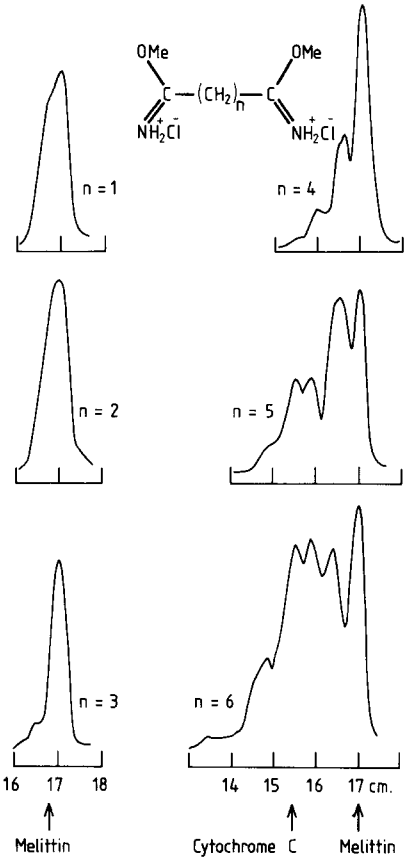

Fig. 5. Comparison of oligomers originating from different cross-linking reagents as determined by polyacrylamide gel electrophoresis. The length of these reagents are given in Table I.

erimidate give higher molecular weight oligomers, including tetramer with a molecular weight of 12000 relative to cytochrome *c* (Fig. 5). None of the reagents gave tetramer alone, and altering the ratio of cross-linker to melittin, had little effect on the composition of oligomers (ratios were generally) less than 5 : 1 by weight, respectively).

The derivatives formed with different reagents were subjected to gel permeation chromatography in both Tris-HCl and phosphate buffers (Fig. 6). They were all found to exhibit similar elution profiles. When native melittin was applied in Tris-HCl (20 mM, pH 7.4) it ran as a broad peak with a molecular weight of approx. 3000. When applied in phosphate (500 mM, pH 7.4) it ran as a tetramer with an apparent molecular weight of 12000 (Fig. 6). These results are consistent with those previously reported [29]. In contrast, although when eluted in Tris-HCl, the cross-linked melittin derivatives showed retention when compared to elution in phosphate, none of the peaks were found to correspond to the native tetramer and without exception the cross-linked material showed absorption behaviour in both Tris-HCl and phosphate solutions. Tetraacetyl- and tetrasuccinyl-melittin ran as tetramers in the Tris-HCl (20 mM, pH 7.4) buffer system, with apparent molecular weights of 11800 and 14200, respectively.

Melittin cross-linked with dimethylpimelimidate resulted in the formation of some tetramer, as determined by gel electrophoresis (Fig. 5, $n = 5$), consequently this conjugation was subject to a more thorough study. Time course analysis of the products (Fig. 7) showed that the reaction was essentially complete after 10 min. Consequently melittin (10 mg) was cross-linked with dimethylpimelimidate for 10 min and subsequently applied to a G-50 column equilibrated with Tris-HCl (20 mM, pH 7.4) in an attempt to isolate the cross-linked tetramer. The first peak of apparent molecular weight 4600 (Fig. 6) was found to consist predominantly of monomer on gel electrophoresis, indicat-

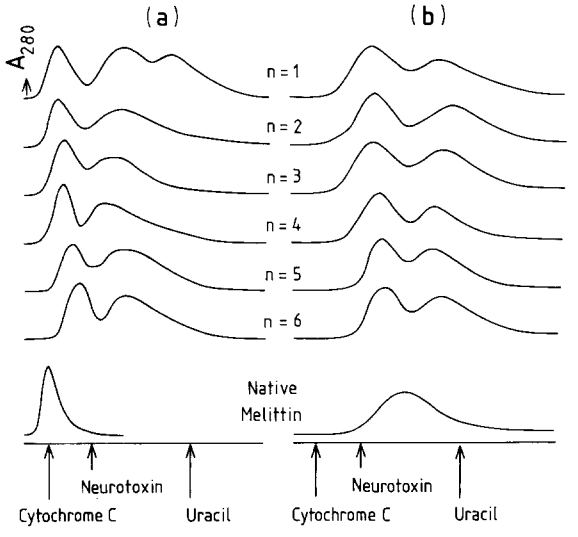

Fig. 6. Comparison of gel permeation (Sephadex G-50 fine, 1.2 × 100 cm) profiles of cross-linked melittin oligomers in (a) 500 mM sodium phosphate (pH 7.4) and (b) 20 mM Tris-HCl (pH 7.4). n = number of methylene groups contained in the cross-link. Melittin and melittin oligomer concentration 2 mg·ml^{-1}.

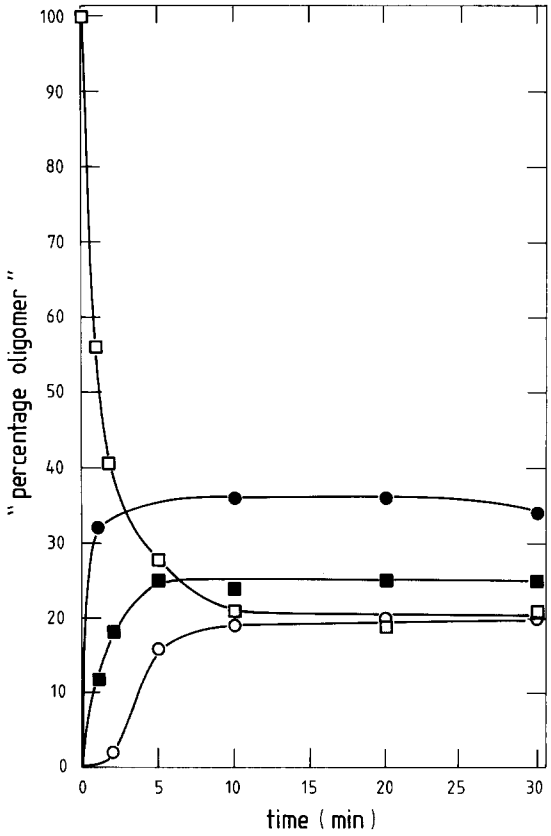

Fig. 7. Variation of the composition of dimethylpimelimidate cross-linked products with time. Data assessed from the intensity profiles of PAGE separations. Melittin monomer (□), dimer (●), trimer (■), and tetramer (○).

ing that the cross-linked tetramer was being retained on the gel, in complete contrast to the native tetramer. It is clear, therefore, that even with dimethylpimelimidate the melittin was not cross-linked in such a manner as to retain the properties of the hydrophilic native tetramer.

The difficulty of cross-linking the native tetramer can be readily explained by the X-ray crystallographic data of melittin tetramer crystals [30]. Calculation of the distances between α- and ε-amino functions in the tetramer give a possible indication of the most favourable interchain cross-links (Table I). Within each dimer (AB and CD) there are considered to be six possible cross-link sites, between A and C three sites and A and D one possible site. Statistically, therefore, the dimer might be expected to be a major product, and indeed this is the case where the reagent is of sufficient length to achieve cross-linking i.e. with adipimate, pimelimidate and suberimidate. The most favourable interchain linking within a dimer is probably between Lys-7 and Lys-21 and yet these residues may also be important in interdimer cross-linking (Table I). If utilised in dimer formation then clearly they will not be available for interdimer interaction. Furthermore, it is possible that formation of a cross-linked dimer changes the relative orientation of the two dimers with respect to each other, thus rendering tight cross-linking of the tetramer more difficult. Under such circumstances a linear array of cross-linked dimers is a more likely product.

TABLE I

DISTANCES BETWEEN α- AND ε-AMINO GROUPS IN THE MELITTIN TETRAMER

In the melittin tetramer a non-crystallographic 2-fold axis of symmetry relates polypeptide chains A to B and C to D. The dimers AB and CD are related by a crystallographic 2-fold axis [25]. Calculation of the distance (Å) between α- and ε-amino functions in the tetramer indicate the positions of the favourable sites of crosslinking (*). A value of 16 Å was chosen as the upper limit for a cross-link site due to the flexibility of lysine residues [25]. (1) Distance (Å) between α- and ε-amino groups in the monomer for intrapeptide cross-linking. (2) Distance (Å) between α- and ε-amino groups in the dimer AB; AB is equivalent to CD. (3) Distance (Å) between α- and ε-amino groups for monomers A and C. The interactions between A and C are equivalent to those between B and D. (4) Distance (Å) between α- and ε-amino groups for monomers A and D which are equivalent to those interactions between B and C. (5) Distance (Å) between imidate centres, i.e. span of cross-linkers.

(1) Distances between amino groups in the monomer

	N^{α}	N_{Lys7}	N_{Lys21}	N_{Lys23}
N^{α}		12.5 *	16.3 *	26.6
N_{Lys7}			25.0	12.5 *
N_{Lys21}				14.0 *

(2) Distances between amino groups in dimer AB

A	B			
	N^{α}	N_{Lys7}	N_{Lys21}	N_{Lys23}
N^{α}	28.1	31.5	14.1 *	15.2 *
N_{Lys7}	32.2	32.0	10.3 *	22.1
N_{Lys21}	15.7 *	11.6 *	22.1	30.2
N_{Lys23}	15.5 *	20.6	28.2	33.8

(3) Distances between amino groups for A to C

A	C			
	N^{α}	N_{Lys7}	N_{Lys21}	N_{Lys23}
N^{α}	38.9	34.0	24.9	38.5
N_{Lys7}	34.0	32.7	18.8	30.9
N_{Lys21}	24.9	18.8	9.4 *	15.7 *
N_{Lys23}	38.5	30.9	15.7 *	24.9

TABLE I (continued)

(4) Distances between amino groups for A to D

A	D			
	N^{α}	N_{Lys7}		N_{Lys23}
N^{α}	33.4	23.0	25.9	32.9
N_{Lys7}	22.2	12.3 *	24.1	27.2
N_{Lys21}	23.1	20.5	19.3	31.0
N_{Lys23}	31.0	24.7	30.3	42.5

(5) Span of cross-linkers

	span (Å)	*n*
Dimethylmalonimidate	3.3	1
Dimethylsuccinimidate	5.0	2
Dimethylglutarimidate	6.3	3
Dimethyladipimate	8.0	4
Dimethylpimelimidate	9.2	5
Dimethylsuccinimidate	11.0	6

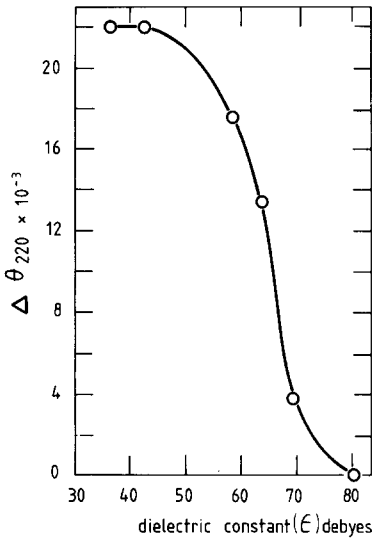

Fig. 8. Effect on the α-helical content of melittin with decreasing dielectric constant. Ellipticity at 220 nm was taken as a quantitative measure of helical content.

(c) Influence of the dielectric constant (ϵ) on the structure of melittin.

The influence of decreasing the dielectric constant on the structure of melittin was measured by circular dichroism, using unbuffered aqueous ethanol solutions (Fig. 8). The results show an increase in α-helical content with decreasing dielectric content, the process reaching completion at $\epsilon = 45$ debyes. The increase in α-helical structure is not accompanied by aggregation [7]. As reported by Bello [27] melittin adopts an α-helical configuration in ethanol rich media. The data previously reported by Dawson et al. [7] was due to the presence of thiocyanate counterions used to enhance the solubility of melittin in non-aqueous media.

Melittin, tetraacetyl- and tetrasuccinyl-melittin were subject to gel permeation chromatography on Sephadex LH-20 in 77% aqueous ethanol ($\epsilon = 36$ debyes) and found to run as monomers (Fig. 9). However, when melittin was applied in 0.5 M ditriethylammonium hydrogen phosphate (TEAP) in 77% ethanol it ran as a tetramer, thus even in media of relatively low dielectric, high concentrations of phosphate favour tetramer formation.

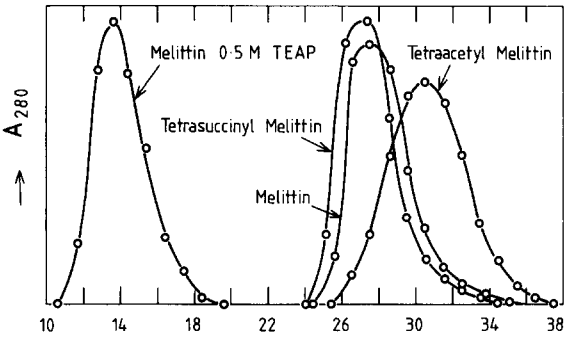

Fig. 9. Gel permeation (Sephadex LH-20, 1.2 × 33 cm) of melittin and melittin derivatives in 77% aqueous ethanol ($\epsilon = 36$) and 77% aqueous ethanol containing 500 mM ditriethylammonium monohydrogen phosphate (TEAP). The column was calibrated as reported by Dawson et al. [7].

Discussion

The influence of melittin on lipid membranes is markedly concentration dependant. Whereas at concentrations above 10^{-6} M it possesses a powerful lytic action [1,31], at sublytic concentrations diverse properties have been reported, including transmembrane channel formation [32] and enhanced ion permeation due to the perturbation of lipid packing [33]. Of the two forms of melittin, it has been suggested that the monomer interacts with cell membranes and is responsible for the lytic activity [7,25]. In support of this concept, enhanced tetramer stability induced by the presence of phosphate renders melittin void of lytic activity (Fig. 3). The hydrophobic portions of the amphiphilic helices are orientated to face one another [25], exposing the hydrophilic residues to the solvent. While these may interact electrostatically with phospholipid head groups, unless the tetramer dissociates, the hydrophobic residues will be unable to insert into the lipid bilayer and induce a wedge effect.

A result which apparently contradicts the concept of the monomer being the lytic agent was presented by Knöppel et al. [24] who reported that a covalently cross-linked tetramer was lytic. In agreement with Knöppel we have confirmed that cross-linked products are indeed lytic, but significantly they retain their lytic activity in high phosphate concentrations (Fig. 2), in complete contrast to the native toxin. Dimethyl suberimidate cross-linked melittin prepared under identical conditions

to those reported by Knöppel et al. [24], was found to possess higher molecular weight oligomers than previously reported. In view of the effects of certain counterions on melittin structure (Ref. 13 and our unpublished data) the results are readily explained. Due to the relatively low concentration of chloride counterions present during the cross-linking of melittin, the peptide was estimated to be 45% α-helical, i.e. 45% tetramer and 55% monomer. Furthermore stopped-flow studies concerning the dissociation of the melittin tetramer yielded a half-life of some 100 ms (unpublished data), indicating that there is rapid exchange between monomer and tetramer. Thus the possibility exists of the tetramer dissociating before both functional groups of the cross-linking reagent can react with adjacent lysine residues. In view of the distribution of oligomers produced and the haemolysis results it would appear that a range of linear oligomers are formed. Even in the phosphate buffer system (0.5 M) where native melittin tends to 100% α-helix, an equivalent structure was not produced from the oligomer. Therefore, in agreement with Knöppel, the products formed by covalent cross-linking are lytic, however, they are not a good model of the native tetramer, which if produced is only a minor product.

It has been demonstrated that melittin in solutions of low dielectric constant adopts a largely α-helical structure (Fig. 8) and yet remains monomeric (Fig. 9). At the membrane interface the dielectric constant is reduced to some 8–10 debyes, compared with a bulk phase value of 80 debyes, due to the presence of structured water associated with the charged lipid headgroups and counterions. The dielectric constant gradually increases through the Gouy-Chapman layer, which can extend up to 30 Å under physiological conditions [34]. As the melittin tetramer has a diameter of approx. 40 Å [36] it can be largely accommodated in this layer. Thus, it is conceivable that the influence of the dielectric constant of the immediate environment of membranes triggers the dissociation of the tetramer. The implication of these studies (Figs. 8, 9) is that the tetramer dissociates to the monomer in the immediate vicinity of the membrane surface and in this environment the monomer retains an α-helical conformation, prior to insertion into the membrane.

Even in solutions at relatively low dielectric constant ($\epsilon = 36$) the presence of high concentrations of phosphate counterions prevents net tetramer dissociation (Fig. 9), thus accounting for the lack of lytic activity of melittin in phosphate buffer, where presumable the Gouy-Chapman layer also possesses elevated phosphate concentrations.

Tetraacetyl melittin, a haemolytic derivative of melittin is tetrameric in aqueous buffer (10 mM Tris-HCl, pH 7.4) but monomeric in solutions of low dielectric (Fig. 9). Thus the presence of the Gouy-Chapman layer readily accounts for the lytic properties of this derivative despite the higher stability of the tetramer. In contrast tetrasuccinylmelittin is non-haemolytic and yet behaves similarly to tetraacetyl-melittin in solutions of low dielectric. This indicates that the lack of lytic activity associated with this derivative is related to its net charge and probably not to the non-dissociation of the tetramer. The entire sequence and structure of melittin would appear to be essential for lytic activity and the cluster of positive charges in the C-terminal region is apparently important. This region contains four positive charges in native melittin and two with tetraacetyl-melittin. In contrast the non-lytic tetrasuccinyl-melittin possesses a net zero charge in this region. It is possibly significant that a homoarginine derivative of melittin is more lytic than melittin [11], indicating that guanidine functions are important for cell lysis. In tetraacetyl-melittin they are unaffected, but in tetrasuccinyl-melittin the strong possibility exists of intramolecular salt bridging between the carboxylate functions of the succinate and guanidine functional groups [35]. Such interaction would decrease the ability of the arginine residues to interact with lipid polar head groups. The possible role of the arginine functions in the direct lytic activity of melittin is currently under investigation.

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