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The interaction of amino-deuteromethylated melittin with phospholipid membranes studied by deuterium NMR

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Melittin, deuteromethylated on each of the four amino groups (Gly-1 *Nα* and Lys-7, 21, and 23 *Nε*), was prepared by reductive methylation using deuteroformaldehyde and $NaBD_3CN$. Deuterium NMR spectra were obtained for the modified peptide (D-melittin) bound to phospholipid bilayers and erythrocyte ghosts. D-Melittin at 4 mol% (peptide:lipid) induced reversible transitions between extended bilayers and micelles at the phase-transition temperature in dimyristoylphosphatidylcholine (DMPC) bilayers. These changes in lipid morphology did not occur at 1 mol% D-melittin: DMPC and the peptide was highly motionally restricted in gel-phase lipid.

Melittin; Deuteration; ^{2}H-NMR; Phospholipid membrane; Vesicle-melittin transition; Chain melting

1. INTRODUCTION

Bee venom melittin has recently been reported to induce reversible transitions between extended bilayers and micelles at the lipid-phase transition in bilayer membranes composed of saturated phospholipids [1–4]. In two of these studies deuterium NMR was used to characterize the interaction of melittin with bilayers composed of acyl-chain-deuterated DPPC [3] and head-group-deuterated DMPC [4]. The effects of melittin on lipid organization in these membrane systems were proposed [3] to underlie the lytic properties of the peptide [5] and to share features of the lysolipid-induced disruption of bilayer membranes composed of saturated lipids [4,6].

Deuterium NMR may also be applied directly to the protein component in protein-lipid complexes using deuterated analogues of the protein [7–9]. We have prepared an amino-deuteromethylated derivative of melittin by chemical modification and show here that some properties of the modified peptide (D-melittin) in lipid membranes (including erythrocyte ghosts) can be determined using deuterium NMR.

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Abbreviations: DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; PL, phospholipase; PS, phosphatidylserine; T_m, gel-liquid crystalline phase transition temperature

2. MATERIALS AND METHODS

Melittin was purified from bee venom by gel filtration on Sephadex G-25 and G-50 [10] and heparin-Sepharose affinity chromatography [11] and was deuteromethylated by reductive methylation [12] using sodium cyanoborodeuteride and deuteroformaldehyde. $NaBD_3CN$ (10 mg; 1.6×10^{-4} mol) were added to a solution of melittin (40 mg; 1.2×10^{-5} mol) in 6 ml of 0.15 M sodium phosphate buffer in D_2O, pH 6.5, containing 2 mM $NiCl_2$, followed by CD_2O (5 mg; 1.6×10^{-4} mol). The solution was stirred at 4°C in the dark for 15 h. A further 5 mg $NaBD_3CN$ and 2.5 mg CD_2O were added and the reaction continued at 18°C for a further 3 h. The solution was then acidified to pH 2 to destroy residual

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$NaBD_3CN$, and the peptide was recovered by gel filtration and lyophilized.

The deuteromethylated peptide was homogeneous by TLC [10] and was unreactive to ninhydrin. Neither *Nε*-dansyllysine nor dansylglycine was detectable by N-terminal analysis [13] at a loading of 50 nmol indicating greater than 98% modification of peptide amino groups [14]. Amino acid analysis, using standard *Nε*-dimethyllysine, showed that the lysines were quantitatively converted to *Nε*-dimethyllysine and that the glycine content was reduced from 3 to 2. These results confirm that melittin was dimethylated on the *Nα*-amino group of Gly-1 and on each of the *Nε*-amino groups of Lys-7, -21 and -23.

Any residual phospholipase A_2 activity in the samples (PLA_2 activity was not destroyed by reductive methylation) was inhibited by using 5 mM EDTA in all buffers, and the absence of lipid hydrolysis was confirmed by TLC after each NMR experiment [4]. The hemolytic activity [5] of *N*-methylated melittin was assayed at pH 7 and was about one-third of the activity of the native peptide at this pH.

DMPC (Fluka), egg PC and bovine brain PS (Lipid Products) were pure by TLC. Samples for NMR were prepared by swelling the lipid in D-melittin-containing buffer made in deuterium-depleted water, to give the required peptide:lipid ratio [4], followed by centrifugation (30 min; 28000 × *g*). D-Melittin was added to human erythrocyte ghosts (approx. 1 mol% peptide:lipid) and incubated at 30°C for 1 h followed by centrifugation as above. Deuterium NMR spectra at 46.1 MHz were obtained on a Bruker WH-300 spectrometer using single 60° pulses of 7 μs [4].

3. RESULTS AND DISCUSSION

Deuterium NMR spectra were obtained for D-melittin bound to bilayers of DMPC, egg PC, egg PC/7 mol% PS and erythrocyte ghosts at a peptide concentration of 1 mol% relative to total lipid (fig.1). Spectra obtained on a high power CXP-300 spectrometer using a quadrupole echo pulse sequence (90° pulse length of 4.5 μs) were similar to those in fig.1, indicating that no components having very large quadrupole splittings or rapid relaxation rates are absent from the spectra obtained on the WH 300 spectrometer.

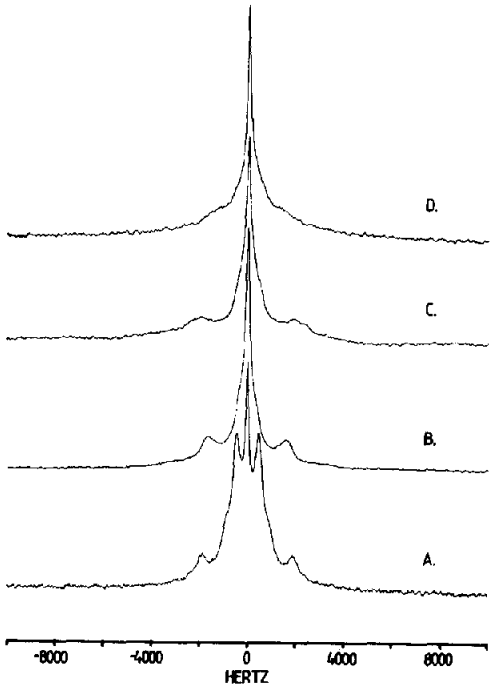

Fig.1. Deuterium NMR spectra (46.1 MHz) of D-melittin bound at 30°C to (A) DMPC; (B) egg PC; (C) egg PC + 7 mol% PS; (D) erythrocyte ghost bilayer membranes at 1 mol% relative to lipid in 50 mM Tris, pH 7.0 containing 5 mM EDTA. 60000–80000 transients were accumulated with 60° pulses (7 μs) and a relaxation delay of 0.12 s.

D-Melittin bound to DMPC bilayers gave deuterium NMR spectra having well-defined quadrupole splittings (fig.1A; top spectrum of fig.2). In egg PC (fig.1B) the spectral components are less well resolved and the contribution of different *N*-deuteromethyl amino groups to the broad and narrow components of the spectrum may not have the same distribution as for the peptide bound to DMPC bilayers. Addition of PS (7 mol%) to egg PC bilayers generates a negative charge at the membrane surface and results in broadening of the deuterium NMR spectrum from membrane bound D-melittin (fig.1C). Comparison with the spectra at temperatures near the phase-transition temperature of DMPC (23°C; fig.2) indicates that this broadening may reflect lowered mobility in the peptide backbone when melittin is bound to negatively charged lipids. The spectrum of D-melittin bound to erythrocyte ghost mem-

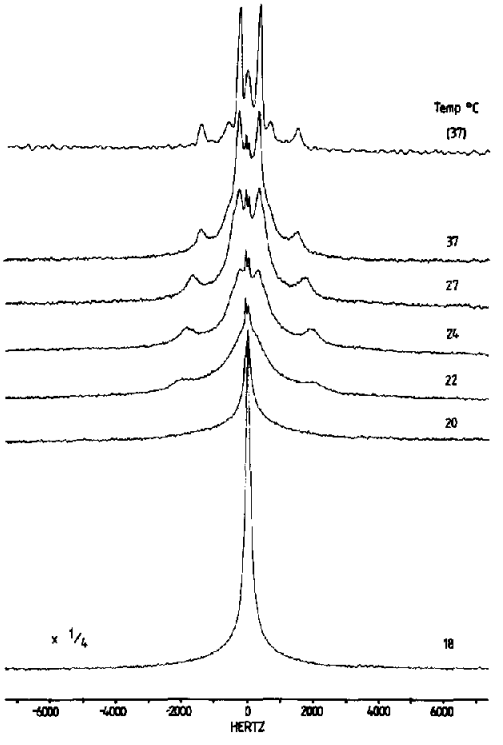

Fig.2. Temperature dependence of the deuterium NMR spectra of D-melittin (4 mol%) bound to DMPC bilayers. In the top spectrum the spectral lines have been artificially narrowed by Gaussian multiplication of the free induction decay, emphasizing the 90° orientational components of the individual powder patterns. The bottom spectrum has been plotted at 1/4 of the total intensity relative to the rest of the spectra. Other conditions as for fig.1.

branes is also broadened (fig.1D) when compared with that from pure phospholipid bilayers in the liquid-crystalline phase, probably due to the non-homogeneous phospholipid composition and the presence of negatively charged lipid in ghost membranes (cf. fig.1C).

Spectra of D-melittin bound to DMPC at a range of concentrations between 1 and 4 mol% are very similar (cf. spectra in figs 1A and 2 at 27°C for D-melittin in DMPC bilayers at 1 mol% and 4 mol% peptide:lipid, respectively) indicating that the component having the largest quadrupole splitting does not arise from self-association of the peptide over this concentration range. Individual spectral components can be resolved by Gaussian multiplication of the free induction decay (fig.2; top spectrum), revealing that the spectrum of D-melittin bound to DMPC consists of components having quadrupole splittings of 3000, 1300 and 600 Hz in relative proportions 1:1:2. (Although the use of Gaussian multiplication for narrowing spectral components is not strictly valid for solid state spectra we have found no distortion of the measured quadrupole splittings with the degree of resolution enhancement used here.) We tentatively attribute the component with the large splitting to the Gly-1 $N\alpha$-deuteromethylamino group since this group, adjacent to the peptide backbone, would be expected to have lower motional freedom than the $N\epsilon$-deuteromethylamino groups of modified lysines. The smaller splittings then arise from the three lysine deuteromethylamino groups, two of which are superimposed to give the splitting of 600 Hz. Similar arguments have been used to assign narrow and broad components of deuterium NMR spectra from deuterated amino acids biosynthetically incorporated into bacteriorhodopsin, to mobile and immobile residues within the membrane protein [9].

D-Melittin partitions completely into DMPC membranes in the liquid-crystalline phase at ratios at least up to 4 mol% as shown by the lack of isotropic components in the NMR spectra due to

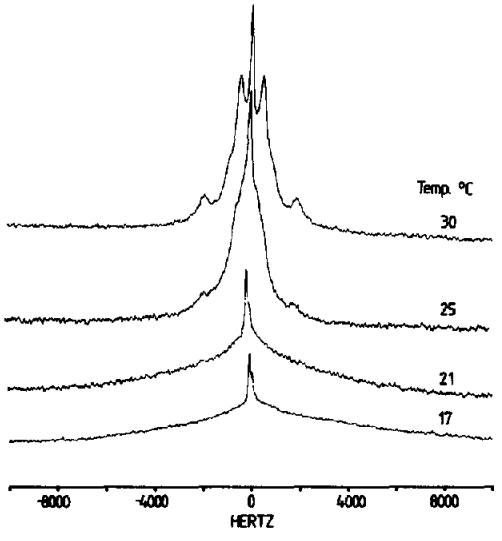

Fig.3. Temperature dependence of the deuterium NMR spectra of D-melittin (1 mol%) bound to DMPC bilayers. Conditions as for fig.1.

free peptide in solution (fig.2). The effect of the lipid-phase transition on the deuterium NMR spectra of membrane-bound D-melittin, illustrated in figs 2 and 3, depends on the ratio of D-melittin to lipid. At ratios of 3 mol% and higher the spectrum of D-melittin bound to DMPC broadens as the temperature is reduced towards T_m (T_m is about 20°C for DMPC containing 4 mol% melittin [4]) and collapses to a single line at lower temperatures. The peptide is now undergoing rapid isotropic reorientations on the time scale of the residual quadrupole splitting, averaging the residual anisotropy to zero. The ^{31}P NMR powder pattern arising from the membrane phospholipid also collapses to a single line under these conditions (not shown) indicating the collapse of the extended bilayer structure to small particles [15], and the transition is associated with a clarification of the lipid suspension. The transition is reversible with spectra indistinguishable from those in fig.2 at temperatures above T_m obtained on rewarming the sample to give liquid-crystalline bilayers. This effect has previously been observed by ^2H and ^{31}P NMR of lipids in melittin-lipid complexes at similar melittin concentrations [2–4], and is associated with a reversible bilayer to disc transition in melittin-lipid complexes composed of saturated lipids [2]. The observation of this phenomenon here indicates that D-melittin interacts with lipid membranes in the same way as the native peptide.

At D-melittin:DMPC ratios below 3 mol% the reversible collapse of the deuterium and phosphorus powder patterns is not observed. Instead the spectra continue to broaden so that below T_m featureless spectra are obtained. This is illustrated in fig.3 for a sample of D-melittin:DMPC at 1 mol%. These spectra illustrate that the mobility of the peptide is highly restricted in gel-phase lipid, the narrow components in the spectra of fig.3 arising from minor amounts of residual HDO and small vesicles as seen by comparing the top spectra of figs 2 and 3. D-Melittin remains firmly associated with the membrane in the gel phase since no isotropic spectral components are observed from free peptide. The complete loss of the spectrum is to some extent an artifact of the requirement for a delay (30 μs) following the non-selective excitation pulse, required to allow time for 'ring-down' of the receiver coil in single pulse spectral acquisition, in which time components having very short transverse relaxation times are lost [16]. The broadening is however a valid indication of a marked decrease in the mobility of D-melittin in gel-phase DMPC bilayers (for a discussion of the possible motional contribution that may generate broadened deuterium NMR spectra see [7,16]).

Finally, we note that the small quadrupole splittings arising from the *N*-deuteromethylamino group of D-melittin bound to DMPC in the liquid-crystalline phase are similar in magnitude to the splittings in head-group-labeled lipids (e.g. the trideuteromethylamino groups of choline in deuterated DMPC [17]). These very small splittings probably indicate rather unrestricted motion of the deuteromethylamines in D-melittin in fluid-phase lipid consistent with their solvation by bulk water at the membrane surface. However, the narrow lines broaden in gel-phase lipid to an extent far greater than observed in deuterium powder patterns of head-group deuterated phospholipid having comparable fluid phase splittings (e.g. DMPC-d_9 [18]). D-Melittin in gel-phase lipid therefore adopts configurations in which the side chains of deuteromethylaminolysines are not freely mobile in the aqueous phase. This conclusion is consistent with the interpretation of Dufourc et al. [3] from a deuterium NMR study of melittin in acyl-chain-deuterated DPPC bilayers that melittin moves deeper into the bilayer as the temperature is lowered through the phase transition into the gel phase.

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