VARIATIONS IN CIRCULAR DICHROISM AND PROTON-NMR RELAXATION PROPERTIES OF MELITTIN UPON INTERACTION WITH PHOSPHOLIPIDS

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Received 2 August 1978

1. Introduction

Melittin, a 26 amino acid cationic peptide extracted from bee venom [1], is well known for its ability to interact with phospholipid structures, dramatically increasing the permeability to aqueous solutes of either natural or artificial membranes [2]. Upon interaction with melittin, the aliphatic chains of the phospholipid molecules seem to undergo a pronounced immobilization, as evidenced by infrared and spin label studies [3,4]. As for the melittin molecule, its only tryptophan residue moves, upon interaction with phospholipids, from an aqueous environment to a hydrophobic one, as judged from the blue shift of its fluorescence maximum [5,6]; the changes in fluorescence characteristics are however dependent on the phospholipid used [6]. No change in the infrared absorption or infrared dichroism spectra of the peptide has been evidenced [4].

We describe here how the intrinsic circular dichroism of melittin and the NMR relaxation properties of its tryptophan protons are modified upon interaction with phospholipids.

2. Materials and methods

Melittin, grade II, phospholipase-free, was purchased from Sigma Chemical Co., St Louis, MO and used as such, dissolved in 0.15 M phosphate buffer (pH 7.4) containing 0.1% (w/v) NaN_3 (in H_2O for the circular dichroism experiments; in D_2O for the NMR experiments).

Lecithin from egg yolk was prepared as in [7]. Dicetylphosphate (from Sigma) was added to egg lecithin in the proportion of 15% (w/w) in order to increase the affinity of melittin for the phospholipids [6].

Phospholipid vesicles were prepared by drying under nitrogen and then under vacuum a chloroform–methanol solution of egg lecithin + 15% (w/w) dicetylphosphate, and letting the residue swell in 0.15 M phosphate buffer, pH 7.4 (in H_2O or in D_2O); the suspension was then sonicated under nitrogen for 3 min at 15 s intervals with an MSE 100 W ultrasonic disintegrator. Mixtures of melittin and phospholipids were re-sonicated before use.

Circular dichroism (CD) spectra were performed at room temperature, using 1 mm path-length cells, on a Cary Model 60 spectropolarimeter with CD attachment. The baseline, given by the buffer alone and/or by the phospholipid suspension without melittin, was subtracted. The mean residue ellipticity, θ, was calculated using mean residue mol. wt 111.5, and expressed as deg. $\cdot$ cm^2 $\cdot$ dmol^{-1}. The performance of the instrument was checked with an aqueous solution of d-10-camphorsulphonic acid.

Fourier transform proton magnetic resonance (PMR) experiments were performed at 29°C. Measurements at 100 MHz were carried out using a Varian XL-100-15 FT Spectrometer, interfaced to a Varian 620/L-100 computer equipped with a 1.24×10^6 word disk accessory. PMR spectra at 270 MHz were obtained using a Bruker WH-270 spectrometer, inter-

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faced to an Aspec 2000 computer. Chemical shifts (in ppm) were evaluated with respect to DSS in D_2O. Proton spin-lattice relaxation times (T_1) were measured at 100 MHz by the inversion-recovery pulse sequence t-180°–τ–90°, τ being the delay between the perturbing (180°) and the monitoring (90°) pulses, and t a long delay time between the application of successive pulse sequences ($\gg 5\ T_1$). In order to minimize the problems arising from the limited dynamic range of the computer, the partially relaxed spectra were obtained under double precision conditions. The accuracy of T_1 values was estimated to be around ± 10%.

3. Results

3.1. *Intrinsic CD of melittin in the absence or presence of phospholipids*

Figure 1 shows the CD spectra of a 0.54 mg/ml

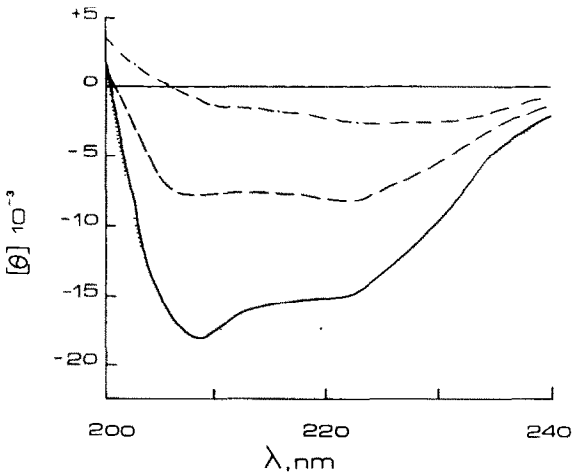

Fig.1. Intrinsic CD spectra of melittin dissolved in aqueous phosphate buffer at 0.54 mg protein/ml, in the absence (——) or presence of phospholipids. The phospholipid/melittin 'molar' ratios were. 2(· · · · · ·); 5 (- - -), 7.5 (- · - · -).

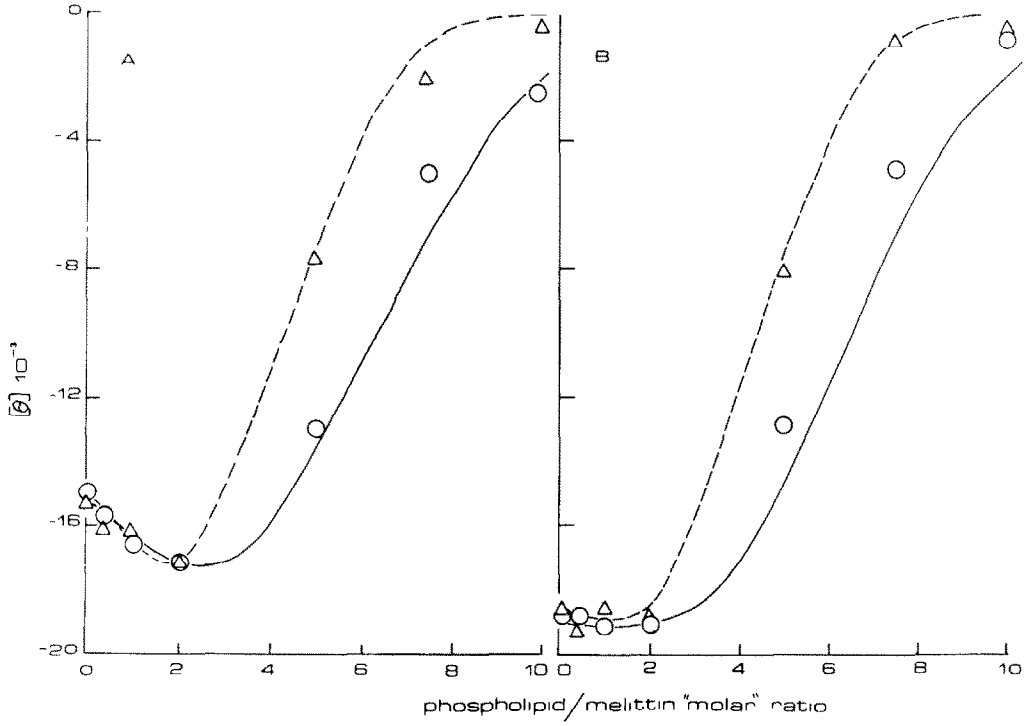

Fig.2. Dependence of melittin ellipticity at 220 nm (A) and at 208 nm (B) on the phospholipid/melittin 'molar' ratio, for melittin solutions at 0.27 mg protein/ml (○) and 0.54 mg protein/ml (△). The continuous and broken lines are the simulated curves obtained for the two melittin concentrations, respectively, as illustrated in section 4, using a θ_{M2}^{220} value of 17.5 × 10^{-3} and a θ_{M2}^{208} value of 20 × 10^{-3} deg. cm². dmol⁻¹.

aqueous solution of melittin, at pH 7.4, either alone or in the presence of 3 different amounts of phospholipids.

Figure 2 illustrates how, for two different melittin concentrations, the ellipticity values of melittin at 220 nm and at 208 nm depend on the phospholipid/melittin 'molar' ratio (i.e., on the number of phospholipid molecules per melittin polypeptide chain). At 220 nm, upon addition of phospholipids we have at first a moderate increase of the absolute ellipticity values, followed, as more phospholipids are added, by a sharp decrease (together with a small shift of the peak toward longer wavelengths). At 208 nm, no variation occurs until a 2:1 molar ratio is attained; thereafter the ellipticity rapidly drops to values around zero.

3.2. *NMR relaxation properties of melittin tryptophan protons*

Figure 3 shows the 270 MHz proton NMR spectrum of the aliphatic region of melittin in phosphate-buffered D_2O: it is apparent that some of the methyl protons undergo remarkable upfield shifts from the main peak (the most shifted resonance appearing at 0.2 ppm from DSS), presumably under the ring current effects originated from a neighbouring aromatic amino acid residue. This result indicates that melittin, in aqueous environment, has a rather folded structure.

In figure 4 is shown the 270 MHz proton NMR spectrum of the aromatic region of melittin under the same conditions (the insert showing the spectrum obtained at 100 MHz, frequency at which the T_1 values of the tryptophan protons were actually determined). Three broad but well-defined signals are observed (signal 1 at ~7.53 ppm, signal II at ~7.42 ppm, and signal III at ~6.96 ppm), which can be attributed to the tryptophan protons. Considering that the area ratios of the peaks (I + II)/III and II/III are about 1.5 and 1.0, respectively (within a 5% approximation), and on the basis of literature data [8,9], the most reasonable assignment is the one indicated in the figure. In particular, although it is difficult to make a definite assignment of the individual protons to the peaks appearing under the profile of the band (I + II), the narrower signal at 7.42 ppm is tentatively assigned to the singlet arising from proton 2, superimposed to a broader signal, probably due to proton 7.

Longitudinal relaxation times, T_1 values, measured at 100 MHz, were found to be 150 ms for signal III and about the same value for signal I; a value of 95 ms was instead found for signal II.

Upon addition of phospholipid vesicles, a broadening of all signals was observed, which, together with the dynamic range saturating effect of the phospholipid alkyl chains, did not allow the study at high phospholipid/melittin ratios. It could however be ascertained that the T_1 value of signal II underwent a definite increase as phospholipids were added, reaching a value of ~300 ms at a phosphatidylcholine/melittin 'molar' ratio of 2 (fig.5). By contrast, the T_1 value of signal III

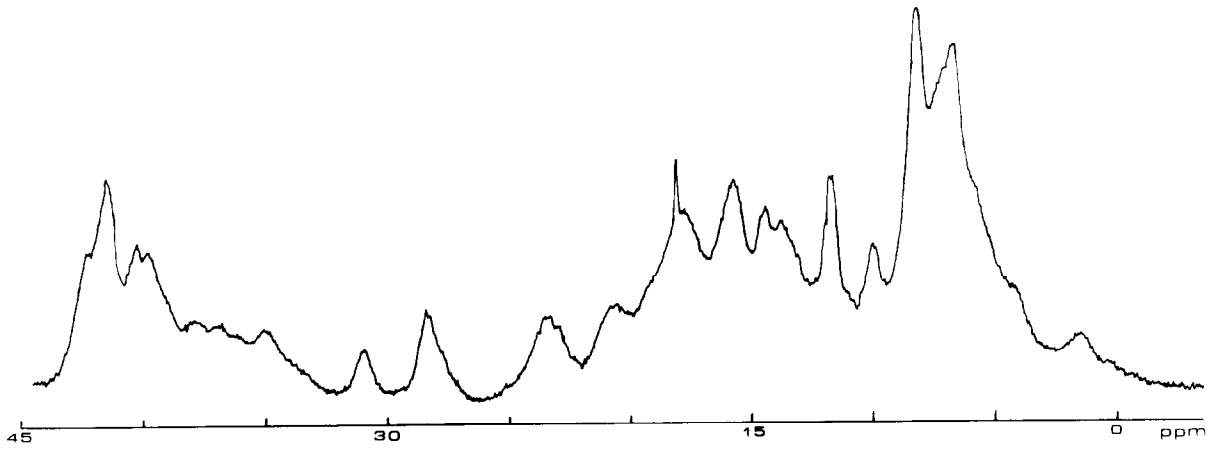

Fig.3. PMR spectrum at 270 MHz of the aliphatic region of melittin at 29°C, 80 mg/ml in 0.15 M ^2H_2O–phosphate buffer, p^{2}H 7.4 (containing 0.1%, w/v, NaN_3); 5 mm (o. d.) sample tube, 1500 transients.

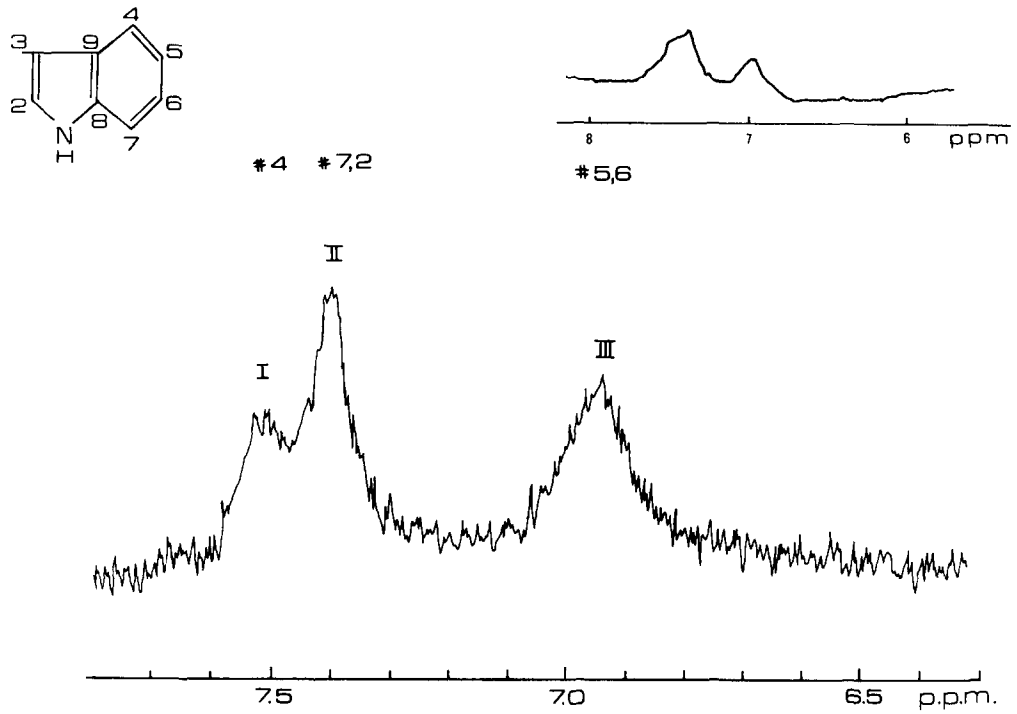

Fig.4. PMR spectrum at 270 MHz of the aromatic region of melittin. Other conditions as in fig.3. Roman numbers indicate the 3 main signals; arabic numbers the tentative assignments to the tryptophan protons. In the insert: proton magnetic resonance spectrum at 100 MHz of the same region: melittin 10 mg/ml; 12 mm (o. d.) sample tube; 1500 transients.

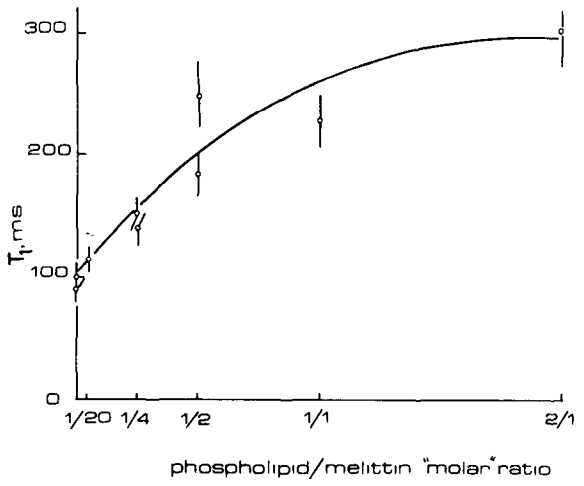

Fig.5. Dependence, on the phospholipid/melittin 'molar' ratio, of the values of the spin lattice relaxation times (T_1) of the tryptophan protons giving rise to signal II of fig.4.

was not significantly changed; variations of the T_1 values of signal I could not be followed, due to the small intensity of this signal and to its partial overlap with signal II.

4. Discussion

It has been reported [10] that, in aqueous solution, melittin presumably exists as a tetramer, in which the hydrophobic amino acid residues are shielded from the aqueous environment. This aggregated structure is probably responsible for the broad signals observed in the PMR spectra reported in fig.3,4, for the exceptionally low T_1 values of the tryptophan protons (especially as far as signal II is concerned), as well as for the relatively low absolute ellipticity values in the region below 230 nm.

Also in agreement with fluorescence data [5], the increase of T_1 values, observed on some tryptophan

protons as a function of the phospholipid/melittin 'molar' ratio (fig.5), indicates that this amino acid belongs to the polypeptide region involved in the interaction with the phospholipids. In particular, the increase of T_1 values observed, upon addition of phospholipid vesicles, for signal II (tentatively assigned to protons 2 and 7) can be attributed to the fact that the interaction with phospholipids disrupts the self-aggregation of melittin molecules. Moreover, the behaviour of T_1 versus phospholipid/melittin ratio can be reasonably interpreted on the basis of the formation of 1:1 complex between melittin and phospholipid.

If the assignments of the NMR signals shown in fig.4 are correct, the interchain aggregation of melittin and its interaction with phospholipids should both concern primarily the tryptophan region between C2 and C7.

The disruption by phospholipids of self-aggregates of melittin (likely leading first to a dimeric form of the peptide and then, possibly, to monomeric ones, although there is no direct evidence in this sense) can also be held responsible for the variations induced by the phospholipids on the intrinsic ellipticity of the peptide. It is indeed well known that the CD spectra of membrane-associated proteins are flattened and distorted by optical artifacts, such as light scattering on the particles, high local absorption and differential light scattering [11,12]. Extensive flattening and some distortion occur also upon aggregation of polypeptide chains [13]. The slight but definite increase in the absolute ellipticity of melittin at 220 nm, observed upon addition of small amounts of phospholipids, could therefore be indeed attributed to the disruption, upon interaction with phospholipids, of the melittin tetramers which are the predominant population in the aqueous milieu. A similar increase in ellipticity has also been found to take place upon association with phospholipids of the myelin protein 'lipophilin' [14] but has been attributed to an increase in α-helix content. Also in our case, especially since the increase of ellipticity was restricted to the 220 nm band, the possibility of a simultaneous slight increase in the α-helix content of melittin, when associated to phospholipids, cannot be ruled out.

The sharp drop in ellipticity occurring, at phospholipid/melittin 'molar' ratios > 3, at 220 nm and even more markedly at 208 nm can instead be definitely attributed to the occurrence, correlated to the insertion of melittin in the phospholipid vesicles, of the above mentioned optical artifacts. This behaviour was typical of melittin, and did not occur when similar amounts of phospholipids were added to other proteins, such as bovine serum albumin.

Within this context we may reasonably assume that the following equilibria hold:

$$M_4 \overset{K}{\rightleftharpoons} 2\,M_2$$

$$L = \alpha \cdot L_\nu$$

$$M_2 + i\,L_2 \overset{K_i}{\rightleftharpoons} M_2L_{2i}$$

where by M_4 and M_2 we indicate tetrameric and dimeric forms of melittin, respectively, by L_ν the phospholipid vesicles (with $\nu = 2700$, according to [15]), by α the fraction (taken to be 0.01) of isolated phospholipid molecules which are not structured into vesicles, and by $2i \geqslant 2$ the number of phospholipid molecules which can be associated to each melittin dimer. Even discarding melittin/phospholipid adducts with $i > 4$, if we attribute to the various association constants appropriate values ($K = 10^5\ M^{-1}, K_1 = 4 \times 10^{19}\ M^{-2}, K_2 = 1.2 \times 10^{36}\ M^{-4}, K_3 = 1.2 \times 10^{54}\ M^{-6}$ and $K_4 = 3.6 \times 10^{72}\ M^{-8}$) and if we assume that $\theta_{M2}, \theta_{M4}, \theta_{M2L2}, \theta_{M2L4}, \theta_{M2L6}, \theta_{M2L8}$ are in a 1:0.825:1:0.5:0:0 ratio at 220 nm and in a 1:0.8:0.85:0:0:0 ratio at 208 nm, a relatively good fit with the experimental CD data, both at 208 nm and at 220 nm and for two different melittin concentrations is obtained, as shown by the continuous lines of fig.2.*

As a conclusion, the NMR and CD spectra both confirm the interaction of melittin with phospholipids. This interaction gives at first an increase of the T_1 values of some of the tryptophan protons (possibly

* The value of $\alpha = 0.01$ stems from the fact that, at critical micellar concentration around 10^{-10} M [16], lecithin forms, in aqueous medium, vesicles of about 2700 phospholipid molecules each. The values of the various association constants and of θ values were instead chosen by repetitive best-fit analysis of the experimental CD result, assuming only that larger aggregates have lower ellipticity values

due to an increased mobility of this aminoacid residue as the protein shifts from an aggregated state to a looser association with phospholipid molecules) and, simultaneously, a small increase of the ellipticity in the 220 nm region. At higher phospholipid/melittin ratios, the intensities of the CD signals in the 230–200 nm region progressively decrease, a fact which can be related to the insertion of the melittin molecules in the phospholipid structures.

Acknowledgement

We warmly thank Professor P. A. Temussi, Centro di Metodologie Chimico-Fisiche, University of Naples, for the use of the Bruker WH270 spectrometer.

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