Biochimica et Biophysica Acta 901 (1987) 101–111
Elsevier

BBA 73599

Incorporation of highly purified melittin into phosphatidylcholine bilayer vesicles

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(Received 17 December 1986)
(Revised manuscript received 30 March 1987)

Key words: Phospholipid bilayer; Melittin; EPR; Lipid–protein interaction; Fluorescence quenching; Energy transfer

Melittin free of phospholipase A_2 was prepared. In the absence of salt this highly pure protein starts to aggregate in solution at a protein concentration of $C_p > 10^{-3}$ M. In high salt solution (2 M) aggregation starts at $C_p > 10^{-6}$ M. This was determined from the blue shift of the intrinsic fluorescence of the protein. Reinvestigation of the quenching behaviour clearly shows that self-aggregation cannot be deduced from quenching experiments using nitrate or 2,2,6,6-tetramethylpiperidine-1-oxyl as quencher. The incorporation of melittin into phosphatidylcholine bilayer vesicles was studied by fluorescence quenching and by energy-transfer experiments using 2- and 6-anthroyloxypalmitic acid as acceptor and peptide tryptophan as donor. Incorporation of melittin into small unilamellar vesicles was found to be reduced below the lipid phase transition temperature, T_t, whereas it incorporates and distributes more randomly above T_t. Cooling the temperature below T_t after incubation at $T > T_t$ leads to a deeper incorporation of the peptide into the lipid bilayer due to electrostatic interaction between the lipid phosphate groups and the positively charged amino acids. This stabilizing effect is lost above T_t and melittin is extruded to the polar phase. Quenching experiments support this finding. EPR measurements clearly demonstrate that even in the presence of high amounts of melittin up to 10 mol% with respect to the lipid broadening of the phase transition curves was only observed with fatty acid spin labels, where the doxyl group is localized near the bilayer surface. The order degree of the inner part of the bilayer remains almost unchanged even in the presence of high melittin content.

Introduction

The amphipathic peptide melittin, the major protein component of bee venom (*Apis melifera*), contains 26 amino acid residues with a large hydrophobic region (amino acids 1–20) and a predominant hydrophilic part at amino acid residues 21–26. Due to this structure melittin interacts with natural membranes and also with artificial lipid bilayers. A single fluorescent residue, Trp-19, is located at the hydrophobic region and therefore it can be used as a sensitive fluorescence probe to

Abbreviations: DMPC, dimyristoylphosphatidylcholine; TEMPO, 2,2,6,6-tetramethylpiperidine-1-oxyl; T_t, transition temperature; 2-anthroyloxypalmitic acid, 2-(9-anthroyloxy)palmitic acid; LUV, large unilamellar vesicles; SUV, small unilamellar vesicles; C_5-spin label, 2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxyl; C_5-spin label ester, 2-(3-carboxypropyl methyl ester)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxyl; C_{12}-spin label, 2-(10-carboxydecyl)-2-hexyl-4,4-dimethyl-3-oxazolidinyloxyl; EPR, electron paramagnetic resonance; I, relative fluorescence intensity.

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study the interaction with membranes.

In solution, melittin is thought to exist in two stable conformations: in a monomeric and in a tetrameric form [1,2]. A change in the conformation of the protein has been reported, which depends on the melittin concentration, the salt concentration and the pH of the solution. Moreover, melittin is considered to be a simple model for protein–membrane associations, and the melittin–phospholipid interaction has been intensively studied by different techniques [3–10].

The biological action of melittin is to enhance the activity of the enzyme phospholipase A_2, another component of bee venom [11]. However, great care has to be taken during melittin purification to separate phospholipase A_2 completely. Several studies have been published using commercial melittin without any purification or only after separation by gel filtration. Due to the similar properties of melittin to phospholipase A_2, in our hands, at least, it is impossible to separate the enzyme by the published procedure, since in this case artifactual results based on the residual activity of phospholipase A_2 will be obtained [12].

The aim of the present paper is to reinvestigate the aggregation behaviour of melittin in solution by fluorescence quenching. Moreover, the incorporation of pure melittin into bilayer membranes exhibiting phase transitions has been studied by fluorescence quenching and by energy transfer measurements. In the crystalline lipid phase the lysine near to the C-terminal end of the peptide was found to interact with the phosphate groups of the lipids [4,13]. However, together with EPR measurement our results support the hypothesis that melittin does not span the membrane, but incorporates near the bilayer surface, maybe in a wedge-like structure.

Materials and Methods

Bee venom from *Apis melifera* (Grade IV) and melittin were obtained from Sigma (München, F.R.G.). All lipids were purchased from Fluka (Neu-Ulm, F.R.G.), checked by TLC and used without further purification. Spin labels were from Syva (Palo Alto, CA) and 2-anthroyloxypalmitic acid was from Molecular Probes (Oregon). The quencher NO_3^- and all other chemicals were obtained from Fluka (Neu-Ulm).

Melittin purification

Preparation of melittin free of phospholipase A_2 was performed by modification of the procedure described by Mollay et al. [11]. 100 mg lyophilized bee venom were dissolved in 20 ml Tris-HCl buffer (100 mM, pH 8.0). 800 µl 2-mercaptoethanol (4 M) were added and the mixture was incubated at 30 °C for 3 h. Then the solution was centrifuged for 7 min at $13000 \times g$, and the supernatant was extracted twice with 2.8 ml *n*-butanol in order to inactivate phospholipase A_2. The aqueous phase was lyophilized, redissolved in 1 ml bidistilled water and loaded to a 50×0.8 cm column of Dowex 1-X2 in the bicarbonate form and chromatographed at 4 °C and at a flow rate of 20 ml/h with bidistilled water. Fractions containing protein were pooled, lyophilized, redissolved in 500 µl bidistilled water and applied to a 30×3 cm column of CM-Sephadex C-25. By elution with 0.5 M NaCl solution other proteins were chromatographed, while melittin was eluted with a 1 M NaCl solution. After lyophilization, melittin was desalted on a 50×3 cm Sephadex G-10 column, pooled and lyophilized again. The desalting procedure was checked by determination of the protein concentration. Amino acid analysis was performed using an automatic amino acid analyser after hydrolysation in 6 N HCl at 110 °C for 24 or 48 h. The overall yield of pure and salt free melittin amounted to 40%.

Phospholipase assay

10 µl of a $5 \cdot 10^{-2}$ M DMPC dispersion (10^{-2} M Tris-HCl, $5 \cdot 10^{-5}$ M EDTA, pH 8.0) were added to 10 µl of 10^{-3} M melittin solution and incubated at 37 °C. Samples were drawn after different time intervals (3–72 h), and analysed by thin layer chromatography on silica gel plates developed in chloroform/methanol/water (65 : 25 : 4, v/v). Original lipid and lysocomponent were made visible with Dittmer/Lester reagent [14]. In addition, radioactively labeled phosphatidylcholine was used. The ^{14}C-label was on the fatty acid chain, which allowed quantitative analysis of the degradation products.

Vesicle preparation

Preparation of small unilamellar vesicles (SUV). 3 mg of lipid were dissolved in 1 ml chloroform, then the organic solvent was removed by a nitrogen stream and by keeping the samples for several hours in a vacuum oven at a temperature above the lipid phase transition temperature. After addition of 1 ml buffer,vesicles were prepared by sonication with a microtip (Branson Sonifier, Cell Disruptor 35, power level 20) above the phase transition temperature for 6 min.

Preparation of large unilamellar vesicles (LUV). These were prepared according to the published procedure of Szoka and Papahadjopoulos [15]. 17 mg DMPC were dissolved in 1 ml chloroform buffer dispersion (2:1, v/v) in a 5 ml conical flask. Then the solution was sonicated briefly (Bandelin Sonorex bath sonifier) until the mixture became a homogeneous dispersion. After 40 min the organic solvent was removed under reduced pressure by a rotatory evaporator. The vesicles were resuspended in 10 ml of buffer solution and washed once by centrifugation to remove small vesicles. Melittin was added to preformed vesicles at the given temperature.

Lipid concentration was calculated from phosphate analysis according to Chen et al. [16]. Melittin concentration was determined from the absorption coefficient $\epsilon = 5540$ at $\lambda = 280$ nm [12] using a Varian Cary 118 absorbance spectral photometer.

Spectroscopic measurements

Fluorescence measurements were carried out using a fluorescence spectrometer Perkin Elmer MPF3. Fluorescence spectra were measured after irradiation at $\lambda = 280$ nm. For the energy transfer experiments the absorption wavelength was set to 280 nm. Emission spectra were taken from 300 to 500 nm. The ratio $(I_{2AP} - I_{2APo})/I_{Mel}$ was determined from the fluorescence maximum of the label at 420 nm in the presence (I_{2AP}) and in the absence of protein. I_{Mel} is the corresponding maximum of the melittin tryptophan fluorescence.

Circular dichroism measurements were performed on a Cary 6002 equipped with the CD accessary. Quartz cuvettes with a pathlength of 0.5 mm were used.

EPR measurements were taken with a Varian EPR spectrometer E 4. The samples were thermostated by a heated stream of nitrogen. Temperature was measured at the sample by a thermocouple. Spectra for C_5-spin label and C_5-spin label ester were analysed by calculating the order degree [17] $S_{app} = (T_{\parallel} - T_{\perp})/(T_{zz} - T_{xx})$ as shown in Fig. 8. Spectra of the more mobile C_{12}-spin label were analysed from the linewidth of the low field peak.

Results

Fluorescence and fluorescence quenching of melittin in solution

Melittin was highly purified. Special care was taken with respect to phospholipase A_2 contamination which is known to be the major impurity [11,18] and, due to the enhancement of its catalytic activity by melittin, the most dangerous. We performed amino acid analysis which showed a close correlation with the theoretically expected values. Phospholipase activity tests using radioactive phosphatidylcholine to determine degradation by thin layer chromatography were performed and compared to commercially pure melittin. Our preparation did not show any degradation superimposed on the background within 72 h whereas commercially available melittin induced detectable lipid hydrolysis within 15 min under the given conditions.

Fig. 1 shows the circular dichroism spectra of the purified and the commercially available melittin. A considerably higher content of α-helices was observed with our preparation.

We reinvestigated the aggregation behaviour of melittin in aqueous solution from the shift of the tryptophan fluorescence maximum. Fig. 2 clearly demonstrates that in the absence of salt a blue shift and therefore probably an association is observed at $C > 10^{-3}$ M. In the presence of 2 M NaCl this blue shift is already observable at a melittin concentration of $C > 10^{-6}$ M.

Vogel [19] determined a salt-induced tetramer formation of melittin by quenching the fluorescence from the aqueous phase. We repeated these experiments using nitrate and TEMPO as quencher molecules and obtained quite different results. Melittin fluorescence was measured as a function of quencher concentration at different

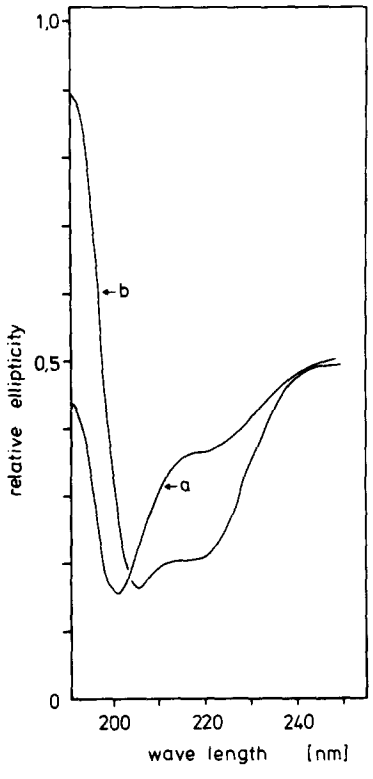


Fig. 1. Circular dichroism spectra of commercially available (a) and purified (b) melittin. Only relative ellipticities are given. The melittin concentration was $3 \cdot 10^{-4}$ M in 10 mM Tris-HCl buffer at pH 7.4. Spectra were taken at room temperature.

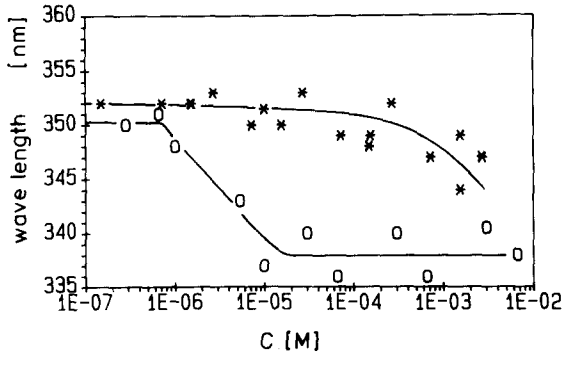
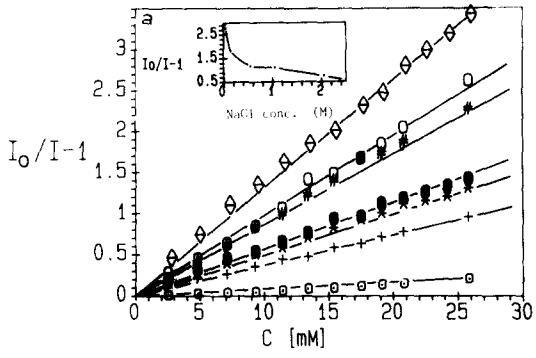


Fig. 2. The maximum wavelength of the melittin tryptophan fluorescence is given as function of peptide concentration in the absence (* ——— *) and in the presence of 2 M NaCl (○ ——— ○). The pH of the solution was 7.4. Spectra were taken at room temperature. Different samples were used, leading to a deviation of ± 3 nm in the determination of the λ_{max} values.

ionic concentrations. Using nitrate as quencher (Fig. 3a) we obtained linear Stern-Vollmer plots following the equation $I_0/I - 1 = K_q \cdot C_q \cdot \tau_0$ where K_q is the quenching constant, C_q the quencher concentration and τ_0 the life time of the fluorophore in the absence of quencher. I_0 and I are the measured fluorescence intensities in the absence and in the presence of quencher. Increasing the salt concentration resulted in a continuous decrease of the quenching constant represented by the slope of the straight lines in Fig. 3a. The lowest quenching rate obtained with melittin



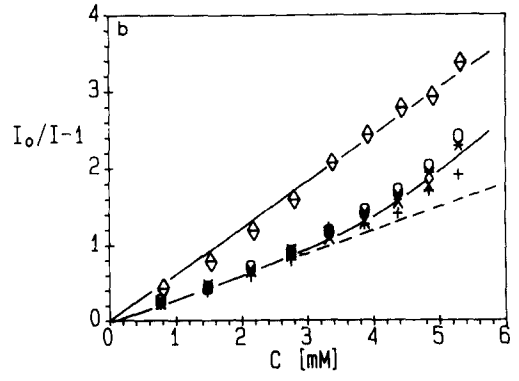


Fig. 3. Quenching of melittin fluorescence at a $8 \cdot 10^{-6}$ M concentration in 10^{-3} M Tris-HCl buffer at pH 7.4. (a) Nitrate was used as quencher. The ion concentration was: (◇ ——— ◇) absence of NaCl; (○ ——— ○) 0.05 M; (# ——— #) 0.1 M; (● ——— ●) 0.5 M; (* ——— *) 1 M and (+ ——— +) 2 M. A quenching curve for melittin in the presence of DMPC vesicles (⊙ ——— ⊙) is given for comparison. The inset gives the dependence of the relative quenching efficiency with increasing ion concentration at a quencher concentration of 15.4 mM. (b) TEMPO quenching at different salt concentrations. The symbols are the same as those utilized in (a).

incorporated into lipid bilayer vesicles is given for comparison. It is important to note that the melittin concentration in the quenching experiments was $8 \cdot 10^{-6}$ M. From the fluorescence maximum at $\lambda_{max} = 336$ nm (e.g., Fig. 2) we know that melittin is aggregated at 2 M NaCl. However, this is not the case at salt concentrations lower than 1 M. At 1 M salt the fluorescence maximum is at 344 nm, whereas at $C \leqslant 1$ M salt we observed a $\lambda_{max} = 350$ nm. Thus, from the continuous decrease in the quenching rate as shown in Fig. 3a it is tempting to conclude that this effect is not correlated with protein aggregation, but should be discussed as a shielding effect, whereby the negative quencher is displaced from the protein surface at high salt concentrations.

Further support of this hypothesis was derived from quenching experiments with TEMPO, an uncharged quencher. As demonstrated in Fig. 3b, a straight line was obtained in the absence of salt. In the presence of salt in concentrations between 0.1 to 2 M the Stern-Vollmer plot is no longer linear but exhibits upward deflection at high quencher concentrations. Quenching curves are almost identical at all salt concentrations even if no multimer formation can be observed in the fluorescence spectra. The upward deflection of the quenching curves gives raise to the assumption of a biphasic quenching mechanism in solution. Quenching in DMPC vesicles at $T = 23\,^{\circ}$C is only slightly lower but linear up to 6 mM TEMPO. Again it is evident that a reduced quenching efficiency is not an indication for a multimeric association but, at least up to 0.5 M salt, simply a shielding effect.

Melittin uptake into SUVs

Primarily we were concerned with the lipid–protein interaction and therefore we have focussed on the incorporation and the distribution of melittin in preformed small unilamellar vesicles. Comparable to the effect during self-aggregation, the incorporation of melittin into lipid bilayers is accompanied by a blue shift of the tryptophan fluorescent due to the decrease in the polarity of the surrounding matrix. If small unilamellar DMPC vesicles were incubated at 4 °C, which is far below the phase transition temperature, melittin incorporated immediately but only partially.

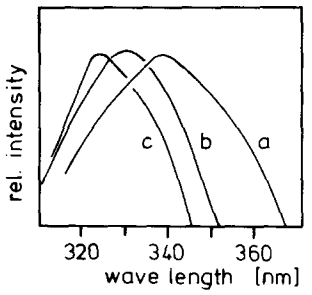


Fig. 4. Fluorescence spectra of melittin added to preformed vesicles after incubation for 15 min at 4 °C (a) at 30 °C (b) and at 4 °C after incubation at 30 °C (c).

This is clearly demonstrated by the fluorescence spectra shown in Fig. 4. If melittin was added at 4 °C we obtained a slightly blue-shifted but broad fluorescence spectrum with a maximum at $\lambda_{max} = 340$ nm. Increasing the temperature to 30 °C lowers the maximum wavelength and the spectrum sharpens. If such a sample was incubated at temperatures above the phase transition of the lipid and was recooled to 4 °C, we obtained a sharpened spectrum with a maximum at $\lambda_{max} = 325$ nm. These experiments clearly demonstrate that melittin is completely incorporated only if the lipid is present at some stage in the fluid crystalline state [5]. The gel state is not able to take up melittin completely from the solution. However, preincubation in the fluid state in the presence of melittin leads to a tight incorporation of melittin into the gel phase.

Large unilamellar vesicles did not show any uptake within a short time (< 15 min) below the lipid phase transition temperature. Even above the phase transition temperature melittin incorporated preferentially into small unilamellar vesicles. This was concluded from the data shown in Table I. Small unilamellar vesicles were preformed and melittin was added. After centrifugation at 13000 $\times g$ we analysed the pellet and the supernatant with respect to phosphate and protein concentration. About 76% of the lipid and also 78% of the melittin content was found in the supernatant, whereas 24% of the lipid and 22% of the melittin were found in the pellet. In the absence of melittin only minor amounts (less than 5%) of lipid sedimented at such a low centrifugal force.

If reversed-phase vesicles were used, the oppo-

TABLE I

DISTRIBUTION OF PHOSPHOLIPID AND MELITTIN IN DMPC VESICLES PREPARED BY ULTRASONICATION (So) AND BY REVERSED-PHASE EVAPORATION(RP)

Vesicles were centrifuged at $13000 \times g$ after melittin addition at $T = 23°C$. The pellet and the corresponding supernatant were analyzed. The vesicle mixture contained a 1:1 molar ratio of phosphate in both types of vesicles. The lipid concentration was $1.6 \cdot 10^{-3}$ M. Data are given as percentages.

	Supernatant melittin	Pellet melittin	Supernatant lipid	Pellet lipid
RP vesicles	17	83	7	93
So vesicles	79	21	76	24
Mixed	57	43	49	51

site situation was observed. In the presence of melittin, 90% of the lipid and 86% of the melittin was found in the pellet. The remaining 10% lipid in the supernatant obviously contained a higher amount of melittin. If SUVs and LUVs were mixed in equal proportion and melittin was added afterwards, we expected an equal distribution of melittin in the supernatant and in the pellet after centrifugation. Lipid was distributed in a 55:45 ratio between pellet and supernatant, which is in good agreement with the expected value. For the melittin, however, we found a distribution that deviates considerably. Only 43% of the peptide was found in the pellet, whereas 57% remained in the supernatant. This suggests that melittin preferentially incorporates into the small vesicles in the supernatant.

Energy-transfer measurements to determine melittin uptake

We investigated the incorporation of melittin into small unilamellar vesicles as a function of temperature by energy-transfer experiments. The tryptophan residue served as energy donor and the anthroyl residue coupled to a stearoyl fatty acid at carbon two (2-anthroyloxypalmitic acid) was used as energy acceptor. In Fig. 5, the energy transfer efficiency $ET = (I_{2AP} - I_{2APo})/I_{Mel}$ is given as a function of temperature. I_{2APo} and I_{2AP} are the fluorescence intensities of 2-anthroyloxypalmitic acid in the absence and in the presence of melittin; I_{Mel} is the fluorescence of the tryptophan residue of melittin.

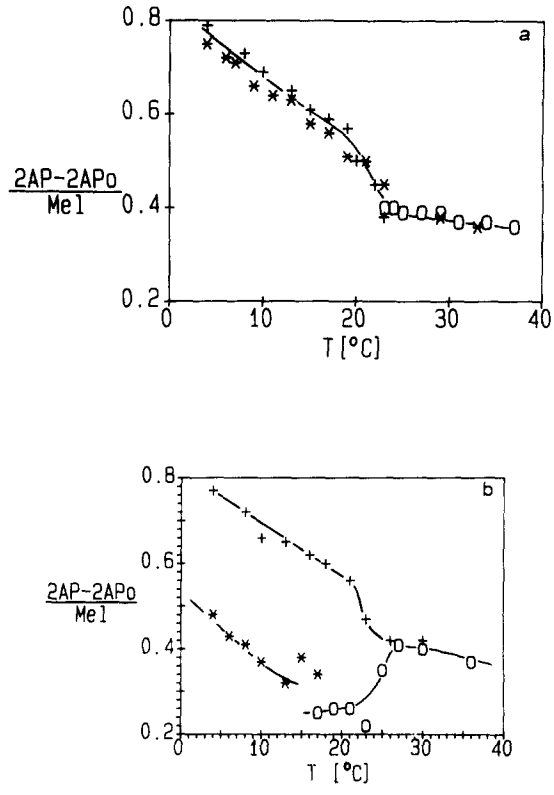


Fig. 5. Temperature dependence of the energy transfer between melittin tryptophan and 2-anthroyloxypalmitic acid. (a) Addition of melittin to preformed vesicles at 23°C. The incubation time was 24 h. Temperature scans were taken starting from 23°C with increasing (O ——— O) and with decreasing (* ——— *) temperature. A second scan starting from 38°C to 4°C (+ ——— +) led to an identical curve. (b) Addition of melittin at 17°C. The sample was split and measured with decreasing (* ——— *) and increasing (O ——— O) temperature. A consecutive scan was taken by lowering the temperature from 34°C (+ ——— +).

If melittin was added to sonicated vesicles at $T = 23°C$, which is at the phase transition temperature, reversible phase transition curves were obtained from energy-transfer measurements. The energy-transfer efficiency is higher at temperatures below the phase transition temperature.

Completely different phase transition curves were obtained if melittin was added at a temperature below the phase transition temperature. Fig. 5b gives an example where melittin was added at 17°C to preformed vesicles. The sample was split in two, one was measured upon cooling, the other was measured upon heating to 35°C followed by

a cooling scan. Addition of melittin at low temperatures first resulted in a lowered energy transfer, which increased at the lipid phase transition. If the phase transition was passed once, the following cooling curve was characterized by an increase in the energy transfer at the phase transition as was also observed for a sample where the protein was added above T_t (e.g., Fig. 5a).

Fluorescence quenching to determine melittin uptake

The temperature-dependent quenching of the melittin tryptophan fluorescence in the presence of DMPC vesicles is shown in Fig. 6. Nitrate was used as quencher and melittin was added at 4° C to preformed SUVs. The high quenching efficiency at low temperature decreased in a step-like manner upon increasing the temperature above the phase transition temperature. Recooling the same sample led to a further decreased quenching rate below the phase transition temperature. At 23.5° C, which is at the phase transition temperature, a small but significant maximum of the quenching was observed.

EPR measurements

EPR spectroscopy was used to study lipid order in DMPC membranes in the presence of melittin. Reversed-phase vesicles were incubated in the presence of melittin above T_t. Vesicles were washed by centrifugation and analysed as described before. Fatty acid spin probes C_5-spin label, C_5-spin label ester and C_{12}-spin label which probe lipid order at different depths within the membrane were used. C_5-spin label is located nearest to the polar surface and therefore exhibits the highest anisotropy of the EPR spectrum at a given temperature. Due to a distortion of the order degree by slow spin-label motion and line broadening, the S-values obtained from EPR spectra as shown in Fig. 7 are apparent.

The C_5-spin label ester is somewhat more mobile due to its deeper incorporation into the bilayer membrane. The doxyl group of C_{12}-spin label is located in the membrane interior and therefore exhibits a highly mobile spectrum which could not be analysed in terms of the order degree but only from the linewidth of the low field peak (Fig. 7). Phase transition curves of DMPC vesicles without protein and vesicles containing 0.5 and 9.5 mol% melittin with respect to the lipid are given in Fig. 8 for C_5-spin label (a), C_5-spin label ester (b) and C_{12}-spin label (c). Surprisingly, it was possible to centrifuge vesicles even in the presence of melittin amounting to 10% with respect to the lipid. The loss of lipid during the washing procedure was only about 5%. From the phase transition curves it is easy to evaluate that melittin in low concentration does not influence

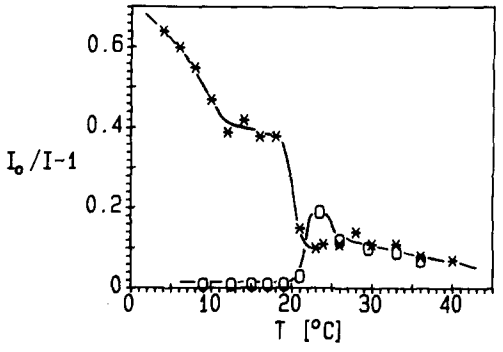


Fig. 6. Temperature dependence of the fluorescence quenching by nitrate ions. Melittin was added at 4° C to preformed DMPC vesicles. The first scan was taken with increasing (* ——— *) and the second consecutive scan with decreasing (O ——— O) temperature.

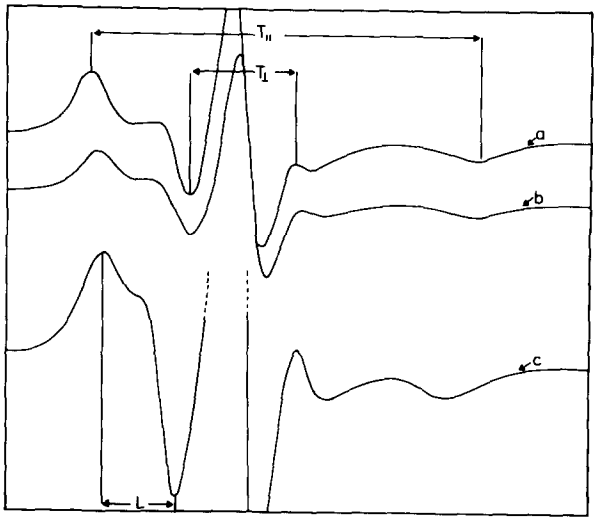


Fig. 7. EPR spectra of C_5-spin label, C_5-spin label ester, and C_{12}-spin label in large DMPC vesicles taken at 13° C. The hyperfine splitting constants $T_{\parallel}$ and $T_{\perp}$, necessary to evaluate the order parameter, are given. Spectra of C_{12}-spin label are analysed by the width of the low field peak.

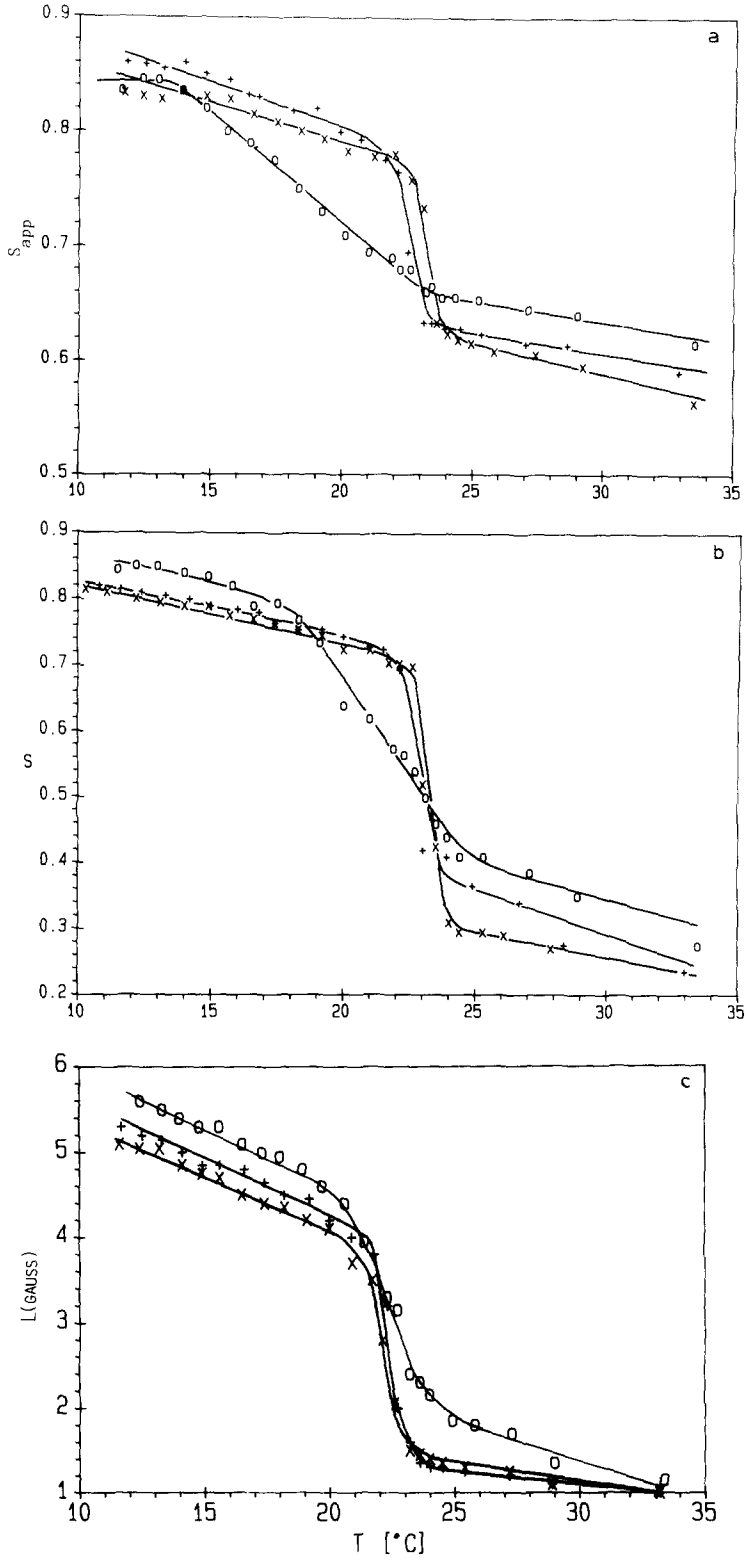


Fig. 8. Phase transition curves of DMPC vesicles without (× ——— ×) and in the presence of melittin (+ ——— + 0.5 mol%, O ——— O 9.5 mol%). Different labels were used: (a) C_5-spin label, (b) C_5-spin label ester, (c) C_{12}-spin label. Spectra for (a) and (b) were analysed by the order degree $S = (T_{\parallel} - T_{\perp})/(T_{zz} - T_{xx})$, and spectra as in (c) were analysed by the linewidth of the low field peak.

the phase transition at all. High amounts of melittin broaden the lipid phase transition preferentially if a probe is used that carries the spectroscopic label near the polar phase. The C_{12}-spin label, even in the presence of almost 10 mol% melittin, does not show a significant change in lipid order.

Discussion

Special care has to be taken if commercially available melittin is used, even if it is labeled as being pure. This has already been noticed recently [18]. Purification by simple gel filtration is not sufficient to avoid lipid hydrolysis [12]. Moreover, from the CD spectra we conclude that additional purification from phospholipase A_2 contamination may drastically influence the conformation of melittin. Our preparation has a considerably higher amount of α-helices compared to those of other authors [2,19]. With such a highly purified peptide we measured the ion strength-dependent multimerization from the blue shift of the intrinsic tryptophan fluorescence. At a melittin concentration of $8 \cdot 10^{-6}$ M, which was used in the quenching experiments, a blue shift was only observed at 2 M salt but not at 1 M salt or less. This is in good agreement with earlier results [20]. Quay and Condie [20], however, did not observe a blue shift in the absence of salt even at high melittin concentrations. Using our melittin preparation the fluorescence maximum decreases from 350 nm to 346 nm between 10^{-4} and 10^{-3} M melittin in the absence of salt. Obviously, melittin aggregation may happen in the absence of salt. From our experiments we suggest that the salt-induced process and the concentration effect is of entropic nature. The chloride ions, which do not induce conformational changes [21], are shielding the positive charges at the lysine and arginine residues. Moreover, water may be displaced from the Helmholtz layer on the surface of the protein, thus making the surface more hydrophobic. Consequently, association occurs via hydrophobic interaction. In the absence of salt, association is hindered by the charge repulsion which has to be compensated by the entropic effect. The gain in entropy, which is the release of surface water, is sufficiently high only at high melittin concentrations in the absence of salt.

Evidence for the exposure of hydrophobic domains in the presence of high salt comes also from quenching experiments. When negatively charged nitrate was used as quencher, we observed a continuously decreasing quenching constant with increasing salt concentration (e.g., inset Fig. 3a) at a given quencher concentration. All Stern-Vollmer plots were linear. One result of these quenching experiments is that it is not possible to interpret reduced quenching rates in terms of multimer formation. From the fluorescence spectra, we definitely know that melittin is not aggregated at salt concentrations below 1 M. It is evident that the chloride ions shield the positive charges on the protein, which inhibits quenching by the low NO_3^- concentration.

TEMPO is soluble in water as well as in apolar solvents, which makes it applicable to the study of lipid phase transitions by EPR spectroscopy. Here we used TEMPO as a fluorescence quencher. In the absence of salt a linear Stern-Vollmer plot was obtained. In the presence of salt we obtained comparable quenching curves at all salt concentrations which exhibit an upward deflection at 4 mM TEMPO. We assume that pure collisional quenching occurs in the absence of salt, leading to a linear Stern-Vollmer dependence. Addition of salt disturbs the water layer at the protein surface and exposes hydrophobic parts to which TEMPO may bind. Additional static quenching will result in an upward deflection of the quenching curves at concentrations as low as 0.1 M NaCl. High salt is not able to displace the apolar noncharged quencher. These deviations from linearity are in agreement with earlier [7] investigations using hydrophobic quenchers.

Primarily we were concerned with the incorporation of melittin into bilayer vesicles. From the blue shift of the tryptophan fluorescence (Fig. 4) we are able to follow the incorporation into unilamellar vesicles. Cooling a sample from above the lipid phase transition temperature to low temperatures resulted in an additional blue shift which we interpret by a deeper incorporation of melittin into the bilayer membrane below the phase transition temperature. Addition of melittin to large unilamellar DMPC vesicles at 4°C did not show

any change in the fluorescence. This confirms the results obtained by Dufourcq et al. [5] who also concluded that no interaction occurs with DPPC dispersions in their gel phase.

A preference of melittin to incorporate into small unilamellar vesicles was concluded from centrifugation experiments. Our results are difficult to interpret in view of the known effect of melittin to induce fusion and morphological changes in small as well as in large vesicles [5]. However, at these very low melittin concentration (0.5 mol% with respect to the lipid) we do not expect dramatic changes. From phosphate analysis of the pellet and the supernatant, we know that with the given melittin concentration 80% of the lipid from small and about 5% of the lipid from large unilamellar vesicles remains in the supernatant, which may be due to the above-mentioned effects.

The importance of the temperature at which melittin is added can also be demonstrated by our energy-transfer experiments (Fig. 5a and b). We conclude that at low temperatures melittin is only partially incorporated into the membrane surface region, leading to a detectable but low energy transfer from tryptophan to the anthroyl group positioned at carbon two of the fatty acid chain. Defect structures that are present below the phase transition temperature [22–25] may be the primary target.

Increasing the temperature above the phase transition temperature leads to an increase of the amount of incorporated melittin and consequently to an increased energy transfer. Now lowering the temperature again leads to a further increase in the energy transfer. We propose that melittin is stabilized in the lipid gel phase by the interaction between the lipid phosphate group and the positively charged amino acids of the protein. This interpretation is backed up by the experimental evidence that inorganic phosphate strongly interacts with melittin [7,21]. We think that this stabilizing effect may bury the tryptophan somewhat deeper into the membrane, which brings it nearer to the C-2 position of the labeled fatty acid. At temperatures above the phase transition temperature the increased lipid headgroup mobility allows the peptide to protrude more into the aqueous phase. This increases the distance to the labeled C-2 position of the fatty acid and thus lowers the energy transfer.

The same interpretation holds for the temperature-dependent quenching experiments shown in Fig. 6. Nitrate was used as quencher, and again DMPC with a phase transition at 23° C is the lipid component. Melittin is added to preformed vesicles at 4° C which yields a high quenching efficiency. This clearly indicates that the melittin tryptophan is located mainly outside the bilayer. However, the fluorescence spectra show a partial blueshift and the ratio $I_0/I - 1$ is decreased compared to melittin in solution, which indicates that part of the added peptide is membrane-bound and probably incorporated into defect structures of the gel phase. As the amount of lipid in defects increases with temperature and especially in the P'_β-phase [23], the quenching rate may decrease continuously due to a further melittin incorporation. At the main phase transition temperature the quenching rate then decreases drastically because of a homogeneous protein incorporation.

A consecutive decreasing temperature scan exhibits the opposite effect. Now the quenching rate drops in a step-like manner at the phase transition with decreasing temperature. In agreement with the energy-transfer measurements, we conclude that melittin incorporates more deeply into the membrane and is shielded by the lipid headgroups from the water-soluble quencher. The maximum of the quenching exactly at the phase transition is probably due to an increased permeability for the nitrate ion, in accordance with earlier studies [25].

In comparison with recent results obtained from NMR spectroscopy [26], our experiments support the model of a wedge-like incorporation of mellitin where both the C- and the N-terminal are located near the membrane surface. In this model, tryptophan at position 19 is well accessible for the 2-anthroyl residue to allow energy transfer. Further support for the idea of a wedge-like incorporation of melittin comes from EPR spectroscopy using fatty acid spin labels to probe different depths in the membrane. The first interesting result is that even if 9.5 mol% of melittin is incorporated, large unilamellar vesicles remain stable and are centrifugable with only small losses (10%) of lipid. We conclude that no lysis occurs. This is in disagreement with the findings of

Dufourcq. The phase transition curves shown in Fig. 9 clearly demonstrate that 0.5 mol% of melittin does not disturb the lipid phase transition behaviour with either label. A strong broadening of the phase transition with membranes containing 9.5 mol% melittin was observed only with C_5-spin label. The effect is less pronounced with C_5-spin label ester and almost not detectable with C_{12}-spin label. To conclude, melittin-induced changes in lipid order mainly occur around the lipid phase transition but only in the apolar phase located near to the head group region. This again fits the hypothesis of a wedge-like peptide structure that incorporates only slightly into one monolayer and does not span the lipid bilayer.

Acknowledgements

This work was supported by a grant from the Deutsche Forschungsgemeinschaft (SFB 169/B2). We would like to thank Mrs. A. Minde for her expert help with the manuscript and Mrs. J. Bendel for her help with the artwork.

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