INCORPORATION OF MELITTIN INTO PHOSPHATIDYLCHOLINE BILAYERS

Study of binding and conformational changes

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1. Introduction

The polypeptide melittin is built up of a (mainly) hydrophobic core covering the first 20 amino acids and a hydrophilic part of 6 amino acids at the carboxylic end [1]. Such a sequence of building blocks is typical for a class of intrinsic membrane proteins such as cytochrome b_5, cytochrome oxidase or glycophorin. Therefore this polypeptide has been used as a simple model to study lipid–protein interactions in artificial lipid membranes [2–5]. It is known that in an aqueous solution of low concentration and low ionic strength melittin adopts a mainly random conformation. Upon interaction with various detergent molecules or lipid membranes a conformational change to a mainly α-helical structure has been observed [4–8]. The binding of melittin to lipid membranes is accompanied by a change of the spectral properties of the single fluorescent residue Trp_{19} [2–5,9,10].

Here, the interaction of melittin with lipid membranes of dimyristoylphosphatidylcholine (DMPC) is studied. Of primary interest are the following questions:

(i) What is the conformation of the membrane bound melittin?
(ii) How is it incorporated into the membrane, i.e., where along the membrane normal is the Trp residue located?
(iii) How accessible is the Trp residue?

Conformational changes of the polypeptide are observed by measuring the circular dichroism (CD). The location of the Trp residue is determined by use of incorporated fatty acid spin labels with a paramagnetic nitroxide group at different positions along the fatty acid chain. The intrinsic fluorescence of melittin is quenched by the spin labels with a higher efficiency the shorter the distance between the Trp residue and the nitroxide group. Quenching of the intrinsic fluorescence by NO_3^-, dissolved in the aqueous phase will probe the accessibility of the Trp residue.

The different types of experiments are performed with DMPC membranes below and above the phase transition temperature (T_t); i.e., with membranes in the ordered and in the fluid phase, to examine whether the properties of the membrane-bound melittin are influenced by the physical state of the lipid membranes.

2. Materials and methods

2.1. *Chemicals*

DMPC was obtained from Fluka, di[1-^{14}C]palmitoylphosphatidylcholine ([^{14}C]DPPC) was obtained from Aldrich, spin labels were purchased from Syva. The lipids were pure as checked by thin-layer chromatography. Melittin was obtained from Merck. Residual phospholipase A_2 activity was removed by gel-filtration through a Sephadex G-100 column [5]. Dispersions of lipid membrane vesicles were prepared by sonication for 30 min at 30°C in the corresponding buffer under a nitrogen gas atmosphere. Buffer: (A) 10^{-2} M NaCl, 10^{-3} M Tris–HCl (pH 7.4); (B) 10^{-1} M NaCl, 10^{-4} M EDTA, 10^{-3} M Tris–HCl (pH 7.4). DMPC/melittin membranes were prepared by adding a solution of melittin in buffer to an equal volume of DMPC membrane dispersion at $T < T_t$ or $T > T_t$, giving a membrane dispersion of the desired concentration and composition.

2.2. *Circular dichroism*

Circular dichroism measurements were performed

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on a Cary 60 equipped with a CD accessory. Quartz cuvettes with pathlengths of 0.5 and 1.0 cm were used.

2.3. *Fluorescence spectra*

Fluorescence spectra were measured on a Perkin-Elmer MPF-3 fluorimeter. Fluorescence polarization measurements were performed on the same instrument equipped with quartz plane-polarizing prisms in the excitation and emission light path. The fluorescence anisotropy is defined as:

$$r = \frac{I^{\parallel} - I^{\perp}}{I^{\parallel} + 2I^{\perp}}$$

$I^{\parallel}$ and $I^{\perp}$ are the fluorescence intensities detected through the analyzer oriented parallel and perpendicular to the direction of polarization of the exciting light.

3. Results and discussion

3.1. *Binding of melittin to DMPC membranes determined by CD measurements*

Interaction of melittin with DMPC membranes causes a change in the CD spectrum (fig.1a) which is explained by a conformational change of the polypeptide from a mainly random structure in aqueous solution to a mainly α-helical form when bound to the membrane. From the value of θ_{222} the fraction f_α of α-helical conformation is determined [11] and summarized in table 1. In the fluid phase f_α is smaller than in the ordered phase. Furthermore, above T_t less lipid molecules are necessary to reach the maximal conformational change. Quantitatively, a melittin molecule, bound to lipid membranes, shows a conformation with ~70% α-helical content corresponding to 18–19 amino acids. According to a Chou-Fasman analysis, the hydrophobic segment of melittin is predicted to be α-helical and $f_\alpha = 0.65$ [6,12] in good

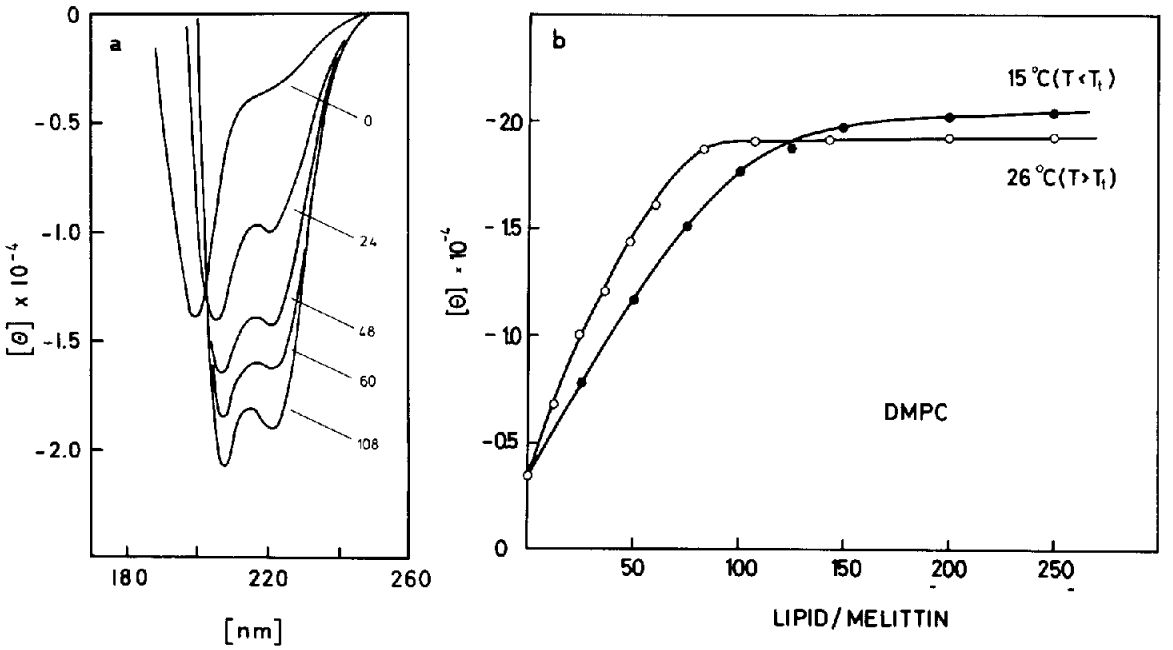

Fig.1. Influence of DMPC membranes on the circular dichroism of melittin, starting concentration 1.14×10^{-5} M at 15°C and 1.19×10^{-5} M at 26°C in buffer A. Increasing amounts of a dispersion of DMPC vesicles (2×10^{-2} M, buffer A) are added to the melittin solution. (a) Changes of the CD-spectrum with increasing lipid/melittin molar ratio. Spectra are corrected for base-line variation and dilution. (b) Change of $\theta_{222} \times 10^{-4}$ with increasing lipid content at 15°C (•) and 26°C (○). Solid lines are the theoretical binding curves using corresponding values of K_B and n as listed in table 1.

Table 1
Spectral properties of melittin

		Circular dichroism[a]				Trp fluoresence[b]			
		$\theta_{222} \times 10^{-4}$[c] [deg . cm² . dmol⁻¹]	f_α[d]	n[e]	$K_B \times 10^{-6}$[f] [mol⁻¹]	λ_{em}^{max} [nm]	Fluorescence intensity	NO_3^- Quenching K_Q [mol⁻¹]	Anisotropy r
Melittin/DMPC (buffer B)	15°C	2.05	0.71	96	2.5	335	1.3	13.8	0.15
	26°C	1.93	0.68	59	3.1	335	1.7	13.8	0.25
Melittin (buffer B)	15°C	0.35	0.07			352	1.0	51.3	0.04
Melittin (2 M NaCl)	15°C		0.65[g]			335	1.0	28.3	0.10

[a] Experimental conditions as in fig.1
[b] Excitation 280 nm; 2×10^{-6} M melittin; 4×10^{-4} M DMPC
[c] In the case of DMPC θ_{222} corresponds to the saturation value of a lipid/melittin molar ratio > n
[d] The fraction of α-helical conformation/melittin molecule is calculated according to [11] with the assumption of 2 helical segments/melittin molecule
[e] Number of lipid molecules/binding place of 1 molecule of melittin
[f] Binding constant K_B determined from titration experiments in fig.1
[g] From [10] at 20°C

agreement with our experimental data with DMPC. Because of the Pro_{14} residue, however, which generally is known to be a helix breaker [12], at least two helical segments exist in a membrane associated melittin molecule. We have observed comparable results with negatively charged lipid membranes of dimyristoylmethylphosphatidic acid ($f_\alpha = 0.72$) and micelles of the detergent SDS ($f_\alpha = 0.74$), indicating that the conformation is rather independent of the lipid headgroup. Our result on SDS agrees with the CD data of detergent–melittin complexes of other groups [6,7], but is in conflict with a value of $f_\alpha = 1.07$ reported in [8].

The CD titration experiments can be interpreted within the following model: In a membrane n lipid molecules (L) represent a binding site for one molecule of melittin (M). For the formation of a lipid–melittin complex (LM) we define a binding constant $K_B = (n\, C_{LM}/C_M C_L)$. C_{LM}, C_M are concentrations (mol/l) of bound and unbound melittin, C_L/n is the concentration of free binding sites. As seen from fig.1b theoretical binding curves fity very well the experimental data. In the fluid membrane phase K is higher and n is smaller than in the ordered phase (table 1). It is known that in aqueous solutions of low ionic strength and at low concentration, melittin exists as a monomer; increasing concentration or ionic strength leads to aggregation of melittin molecules in the form of tetramers with a change to $f_\alpha = 0.65$ [10], a value similar to the one observed in lipid membranes.

In an independent gel filtration experiment we examined whether the conformational changes of melittin upon interaction with lipids are caused by binding to the membranes. Fig.2 shows that in a DMPC–melittin complex of molar ratio $m = 200$ no free melittin is detected; all the melittin emerges together with the membrane vesicles, both below and above T_t. This result proves that melittin is tightly bound to the DMPC membranes.

3.2. *Intrinsic fluorescence*

An aqueous solution of monomeric melittin exhibits an intrinsic fluorescence with an emission maximum $\lambda_{em}^{max} = 352$ nm. In the presence of DMPC membranes ($m = 200$) λ_{em}^{max} shifts to 335 nm, together with an increase of the fluorescence intensity (table 1). The fluorescence intensity Q of melittin in the membrane-bound state relative to the intensity Q_o in water solution has a value of $Q/Q_o = 1.3$ at $T < T_t$ and $Q/Q_o = 1.7$ at $T > T_t$. The temperature dependence of Q/Q_o shows a break at T_t, similar to the case with dipalmitoylphosphatidylcholine (DPPC) [9].

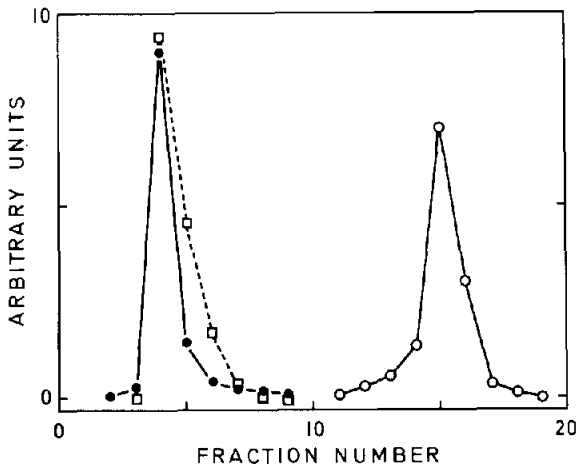

Fig.2. Gel filtration of melittin. Samples of 0.25 ml are applied to a 20 × 0.6 cm Sepharose 4B column in buffer B, 2×10^{-4} M melittin, 4×10^{-2} M DMPC. A small amount of [^{14}C]DPPC is added to DMPC before sonication. Lipid concentration in the effluent is determined by measuring the content of [^{14}C]-DPPC; melittin concentration is analyzed by the absorbance at 280 nm and by the intrinsic fluorescence: (□) DMPC membrane vesicles; (○) melittin; (●) DMPC/melittin membranes; the composition of the applied sample and the effluent is identical with m = 200.

In an aqueous 2 M NaCl solution melittin exists as a tetramer, with λ_{em}^{max} = 335 nm and unchanged fluorescence intensity Q_0. From these results we conclude that the aggregation process monomer → tetramer and/or the conformational change random coil → α-helix induces a shift of λ_{em}^{max} from 352–335 nm, whereas binding of the polypeptide to a lipid bilayer in addition increases the fluorescence intensity to an amount which depends on the physical state of the bilayer. An increase of the fluorescence intensity by a factor of 1.7 together with a blue shift of the emission maximum can be simulated when melittin is dissolved in a dioxan/water mixture with a dielectric constant ϵ = 30. This might support the idea that the Trp residue of membrane-bound melittin is located in the region of the glycerol backbone of the lipid molecules where $\epsilon \approx 30$.

3.3. *The location of the Trp residue in the lipid bilayer – Fluorescence quenching by fatty acid spin labels*

Paramagnetic probes are known to quench the fluorescence of fluorophores [13,14]. We have used fatty acid spin labels with the paramagnetic nitroxide group at carbon atom 1 (SL1), 5 (SL5) and 12 (SL12), in order to quench the tryptophan fluorescence in DMPC–melittin membranes (m = 200) at $T \gtrless T_t$. The results are shown in fig.3 and can be summarized as follows:

1. The quenching process is described by the Stern-Vollmer relationship $(I_0/I)-1 = K_Q c$, where I_0 is the fluorescence intensity without quencher, I is the fluorescence intensity in the presence of quencher of concentration c, K_Q is the quenching constant.

2. The quenching efficiency increases in the order SL12 < SL5 < SL1, both in the fluid and in the

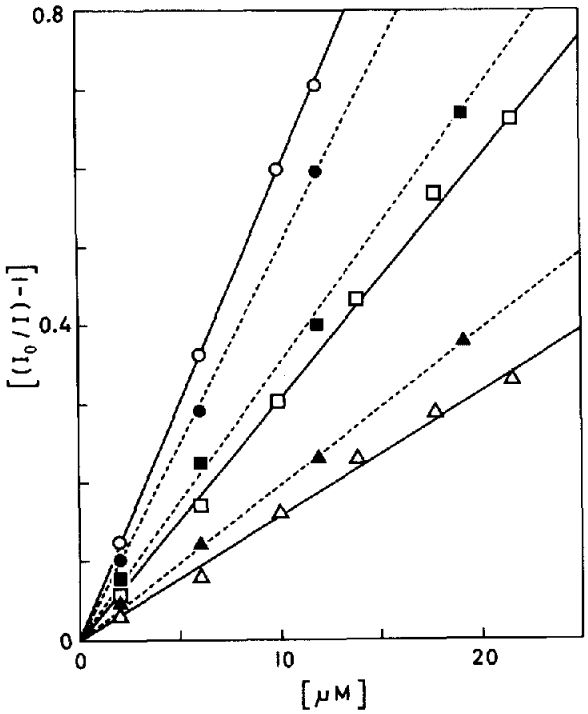

Fig.3. Quenching of the Trp fluorescence of DMPC/melittin membranes of molar ratio m = 200 by spin labels: 4-(dodecanoyloxy)-2,2,6,6-tetramethyl piperidine-1-oxyl (SL1); 2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxyl (SL5); 2-(10-carboxydecyl)-2-hexyl-4,4-dimethyl-3-oxazolidinyloxyl (SL12). Appropriate amounts of spin-labels, dissolved in tetrahydrofuran (THF) are added to an aqueous dispersion of membranes. Final concentration of THF was <2%: (○) SL1, (□) SL5, (△) SL12 at 15°C ($T < T_t$), solid lines; (●) SL1, (■) SL5, (▲) SL12 at 30°C ($T > T_t$), broken lines; 2×10^{-6} M melittin, 4×10^{-4} M DMPC, buffer B.

ordered membrane phase. This result implies that the vertical location of the Trp residue is between carbon 1–5 of the spin label, i.e., in the region of the lipid glycerol backbone.

3. The quenching constants $K_Q \times 10^{-3}$ of SL12, SL5, SL1 are 19.7, 35.3 and 50.0 mol^{-1} at 30°C and 15.8, 30.8 and 60.2 mol^{-1} at 15°C. The relative difference between the K_Q values above and below T_t indicates that the Trp residue changes its vertical position when going from the fluid to the ordered membrane phase. Above T_t the Trp residue is buried deeper in the membrane than below T_t.

3.4. *Accessibility of the Trp residue from the water phase – Fluorescence quenching by NO_3^-*

The intrinsic fluorescence of melittin can be quenched by NO_3^- as shown in fig.4. The quenching constants deduced from the Stern-Vollmer plots decrease in the order monomer > tetramer > membrane bound melittin (table I). In general, the quenching constant $K_Q = k\tau$ is a product of rate constant k for collision-induced quenching and the life-time τ of the excited state [15].

No appreciable difference of τ between the monomer, tetramer and membrane bound melittin was observed (L. Best, unpublished). A decrease of K_Q is therefore attributed to a decrease of k, corresponding to a lower accessibility of the Trp residue. In a aqueous solution of monomeric melittin, the Trp residue is highly accessible by NO_3^- (K_Q = 51.3 mol^{-1}) very similar to the case of an aqueous solution of indole (K_Q = 48 mol^{-1}) [16]. A solution of tetrameric melittin in water shows a reduced accessibility of the Trp residue (K_Q = 28.3 mol^{-1}), due to the aggregation and/or conformational change. The incorporation of the polypeptide into a lipid membrane is accompanied by a further protection of the Trp residue (K_Q = 13.8 mol^{-1}).

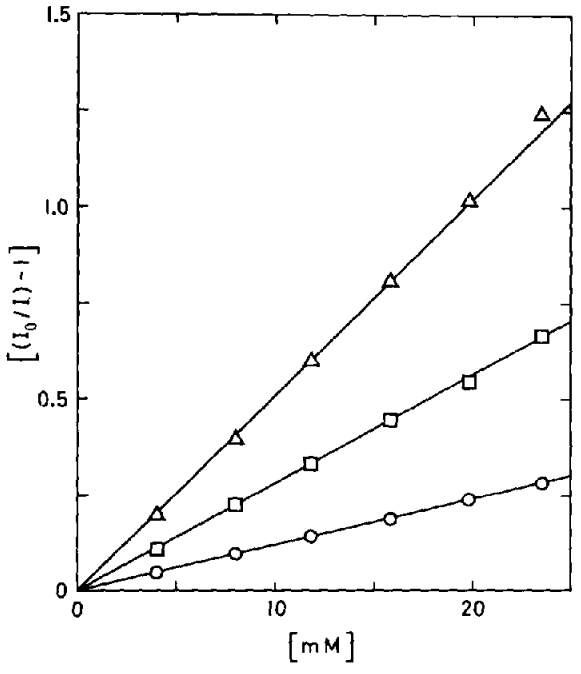

Fig.4. Quenching of the Trp fluorescence of melittin by $NaNO_3$. Appropriate amounts of an aqueous solution of 2 M $NaNO_3$ are added: (△) 2 × 10^{-6} M melittin, buffer B, 15°C; (□) 2 × 10^{-6} M melittin, 2 M NaCl (pH 7.4); (○) DMPC/melittin = 200, 2 × 10^{-6} M melittin, buffer B; within experimental error identical values are obtained below and above T_t.

4. Conclusion

Our experiments have shown that melittin binds strongly to lipid membranes thereby changing its conformation from a random coil structure in aqueous solution to a 70% α-helical form in the membrane-bound state. The binding is influenced by the physical state of the lipid membrane.

A higher binding constant is found in the fluid than in the ordered membrane phase. The number of lipid molecules constituting a binding site in the membrane is n = 96 below T_t and n = 59 above T_t. Assuming the α-helical part of melittin to be a cylinder with diam. 12.5 Å, penetrating the lipid bilayer, lipid molecules from the cylinder surface up to a distance of 21 Å ($T < T_t$) and 18 Å ($T > T_t$) are affected by the polypeptide.

From the quenching studies it was found that the Trp residue of the membrane-bound melittin is located in the region of the glycerol backbone of the lipid molecules with a lower accessibility compared to the aqueous solution. This finding is in agreement with the result of an NMR study of a melittin–lysophosphatidylcholine complex [17] and furthermore with the penetration of melittin into the lipid bilayer as determined by crosslinking experiments [18]. At the phase transition the Trp residue slightly changes its position: above T_t it is buried deeper in the lipid bilayer than below T_t. This observation explains the increase of the Trp fluorescence intensity and aniso-

tropy r, when going from the ordered to the fluid membrane phase. A deeper penetration of the Trp residue into the lipid bilayer causes its microenvironment to be more hydrophobic (lower ϵ) and therefore increases the fluorescence intensity. In addition, the rotational mobility is lowered as indicated by an increase of r, although this mobility change may also arise partially from a change of the conformation and aggregation of melittin. A vertical displacement of membrane proteins in human erythrocytes mediated by changes in microviscosity have been described, which may be a regulating mechanism in the interaction of various ligands with their membrane receptors [19].

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