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^{13}C-NMR Investigation of the insertion of the bee venom melittin into lecithin vesicles

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Abstract. The orientation of melittin in lecithin membranes was investigated by means of ^{13}C-NMR spectroscopy. Phospholipase-free melittin was labeled with ^{13}C-methyl groups at the ε-amino side chains of lysine 7, 21, and 23. From the pH dependence of the corresponding ^{13}C resonances, pK values of the lysine residues were derived that were different for free and membrane-bound melittin. The shift reagent $Pr(NO_3)_3$ induced shifts in the ^{13}C resonance position of all three lysines when melittin and the shift reagent were added to a lecithin vesicle suspension, whereas Pr^{3+} ions included in the inner volume of the vesicles did not affect the ^{13}C resonances of melittin bound to the outer vesicle membrane. A wedge-like structure was derived for the membrane-bound melittin, the lysine side chains of which are freely accessible to the aqueous solvent.

Key words: Melittin, membrane-bound protein conformation, ^{13}C-NMR.

Introduction

The polypeptide melittin is the main component of bee venom. Its membranolytic function is apparently related to its detergent-like character. The amino acid sequence starts in the N-terminal part with 20 predominantly hydrophobic amino acid residues and finishes with 6 charged and hydrophilic residues in the C-terminal part (Habermann and Jentsch 1967):

+Gly-Ile-Gly-Ala-Val-Leu-Lys(+)-Val-Leu-Thr-
-Thr-Gly-Leu-Pro-Ala-Leu-Ile-Ser-Trp-Ile-
-Lys(+)-Arg(+)-Lys(+)-Arg(+)-Gln-Gln.

The amphipathic character renders melittin water-soluble and enables it to integrate spontaneously into membranes. According to an X-ray analysis the structure is predominantly α-helical with a bend near the proline-14 residue. In aqueous solution a tetramer is formed out of four identical subunits; the dissociation constant of the tetramer is dependent on the pH and on ionic strength (Brown et al. 1980; Quay and Condie 1983). In many investigations melittin has served as a model system for protein-lipid interactions, although the orientation of the polypeptide in a membrane system has not yet been clarified. The question arises as to whether the charge of the lysine residue in position 7 prevents an integral insertion of the hydrophobic sequence 1 to 20 into the membrane. Since the tertiary structure of melittin crystallized from an aqueous solution is known, it should be interesting to obtain information about conformational changes occurring during the insertion process.

The aim of this investigation was to describe the orientation of melittin inserted into phospholipid vesicles, using high-resolution NMR spectroscopy. By introducing ^{13}C spin labels at either end of melittin it should be possible to detect the accessibility of the C-terminal or the N-terminal part to the solvent inside or outside the phospholipid vesicle.

Materials and methods

Preparation of melittin

Melittin was isolated from bee venom according to Maulet (Maulet et al. 1982). Lyophilized bee venom (obtained from Roth, Karlsruhe, Germany) contains approx. 50% melittin and besides other proteins and peptides approx. 1% phospholipase A_2 (EC 3.2.2.4). In order to investigate the interaction of melittin with phospholipid membranes it is necessary to

* To whom offprint requests should be sent
Abbreviation: NOE Nuclear Overhauser Enhancement

carefully exclude the phospholipase A_2. Since phospholipase A_2 contains three methionine residues, whereas melittin does not contain any methionine, the phospholipase A_2 could be split by cyanogen bromide and the fragments could be easily separated from the melittin. Prior to the cyanogen bromide fragmentation the disulphide bridges were split under reductive conditions and the SH groups were blocked by *p*-hydroxymercuribenzoic acid. Subsequent ion exchange chromatography and a final gel chromatography step separated melittin from the peptide fragments of phospholipase A_2.

Preparation of ^{13}C-labeled dimethyl-lysyl-melittin

In order to investigate melittin with ^{13}C-NMR spectroscopy in the presence of large surplus amounts of lipids, a ^{13}C-labeled derivative was prepared. Since the introduction of a label should not lead to a change in the chemical properties of melittin, in particular not in the number of charges in the protein, a reductive alkylation with ^{13}C-formaldehyde was chosen to introduce two methyl groups at the ε-amino groups of lysine 7, 21, and 23 (Means and Feeney 1968).

For the reaction approx. 15–20 mg lyophilized melittin were dissolved in a borate buffer of pH = 9.0. At time intervals of approx. 5 min equal volumes of ^{13}C-labeled formaldehyde were added up to a concentration at which the alkylating reagent was approx. double the concentration of melittin. The reductive condition during the reaction was maintained with the addition of dissolved sodium borohydride. After 30 min the reaction was stopped by the addition of acetic acid, which brought the pH to a value of 5.0. This solution was neutralized and the modified melittin was separated from the reactants with gel chromatography (Sephadex G 25 f).

Preparation of lecithin liposomes

Vesicles were prepared either from synthetic dimyristoyl-lecithin (DMPC obtained from Calbiochem, Frankfurt, Germany) or from egg yolk lecithin (obtained from Lipoid KG, Papenburg, Germany). Thin-layer chromatography was used to check that the DMPC was essentially free of impurities. The egg lecithin contained approx. 1%–2% fatty acids, lysolecithin, or oxidized lecithin; it was therefore further purified on a silica gel chromatography column. Unilamellar lecithin vesicles were prepared from a 20–100 m*M* lecithin suspension. The lecithin was first dispersed for 5 min with a shaker and then sonified for 1–2 h with a Branson sonifier. Trace quantities of titanium debris from the sonifier were eliminated from the clear but slightly opalescent vesicle suspension by centrifugation. Vesicles containing Pr^{3+} ions in the inner volume were prepared in the same way from a Pr^{3+} solution. The Pr^{3+} ions in the outer medium were removed by using a chelating ion exchange resin (Chelex 100, BioRad Laboratories, Richmond, Calif., USA).

The average size of the vesicles was determined by gel chromatography on Sephacryl S 1000 (Pharmacia Fine Chemicals, Uppsala, Sweden). The column (50 × 1.5 cm) was calibrated with latex particles of a defined size (106 nm, 175 nm, and 310 nm diameter) suspended in a 2% Triton X-100 solution. The size of the eluted particles was found to be proportional to their K_{AV} value. The K_{AV} is defined as:

$$K_{AV} = \frac{V_{max} - V_0}{V_{bed} - V_0}, \quad (1)$$

V_{max}: Elution volume of the particles at the maximum value of E_{300}
V_0: Excluded volume of the column
V_{bed}: Bed volume of the column.

Reproducible V_{max} values were found for the vesicle preparations; the diameter of the unilamellar lecithin vesicles was determined to be 20 ± 2 nm. An almost symmetrical maximum was found in the elution profile. The elution diagrams revealed a narrow and homogeneous size distribution.

Upon the addition of Pr^{3+} ions to the outer medium of the vesicles, ^{31}P or ^{13}C resonances of the outer sphere lipids of the bilayer membrane can be separated from those of the inner monolayer.

NMR measurements

NMR investigations were carried out at 67.93 MHz with a 270 MHz AM Bruker spectrometer, which was equipped with an Aspect 3000 computer system. The samples contained approx. 20% ^2H_2O for the lock signal. In a typical ^{13}C-NMR experiment a pulse of 15 µs was chosen. The acquisition time was around 0.5 s. The use of the WALTZ pulse sequence allowed an effective proton decoupling of the ^{13}C resonances without detectable heating of the NMR probe. Owing to NOE effects, the intensity ratios of the ^{13}C signals were not disturbed since decoupling was used only during the acquisition. The inversion recovery sequence was chosen to determine the spin-lattice relaxation times of the labelled carbon resonances. The pH values were determined within the NMR tubes by using a combined electrode (Ingold, Frankfurt, Germany).

The reported pH values were pH-meter readings without a correction for the deuterium isotope

effect. Usually the solutions were 0.1 *M* in sodium chloride and 0.1 *M* in sodium borate. For the evaluation of pK values from the pH dependences of the chemical shifts, the Henderson-Hasselbalch equation with a weighed Hill coefficient, n_i, was used,

$$\delta_{obs} = \delta_{min} + \sum_i \Delta\delta_i \cdot \frac{10^{(pK_i - pH)\cdot n_i}}{1 + 10^{(pK_i - pH)\cdot n_i}} \quad (2)$$

in which δ_{obs} is the observed chemical shift value and $\Delta\delta_i$ is the difference of the chemical shifts between protonated and unprotonated species. By means of a computer fit program, a titration curve was fitted to the experimental values, with $\Delta\delta$, pK_i, and the n_i values used as variables.

Results

pH dependence of the ^{13}C-labeled aminomethyl resonances of modified melittin in aqueous solution

Under the conditions chosen (10 m*M* melittin, 0.1 *M* sodium borate, 0.1 *M* sodium chloride) melittin had a tetrameric structure. It would have been difficult to follow the protonation equilibria of the side chains of monomeric melittin, since the association constant of the tetrameric complex increases with deprotonation of the basic side chains (Quay and Condie 1983; Brown et al. 1980). In order to avoid an overlap of protonation equilibria with the complex formation, a concentration of melittin of about $5 \cdot 10^{-4}$ mol/l should not be exceeded. However, this would have required very long acquisition times for the NMR spectra. Therefore conditions were chosen such that the melittin remained tetrameric throughout the investigated pH range. In the course of titration a reversible pH dependence of the chemical shift of the ^{13}C resonances between pH = 7 and pH = 11 was observed. At pH values lower than pH = 7 a sharp resonance signal appeared in the spectrum at 44.1 ppm. The chemical shift of this resonance corresponds to the chemical shift value of the methyl resonances of Nε-dimethyl lysine (Baxter and Byvoet 1975). At higher pH values this resonance splits into one larger, broader signal *A* and one smaller signal *B*. In Fig. 1a both signals are plotted for different pH values. At even lower field an additional weak signal was observed. This resonance is pH-dependent below pH = 8. It was assigned to the ^{13}C-methyl resonance of the N-terminal α-amino group of melittin, which was expected to be only slightly methylated. Since resonances *A* and *B* overlap considerably, a Gaussian filtering of

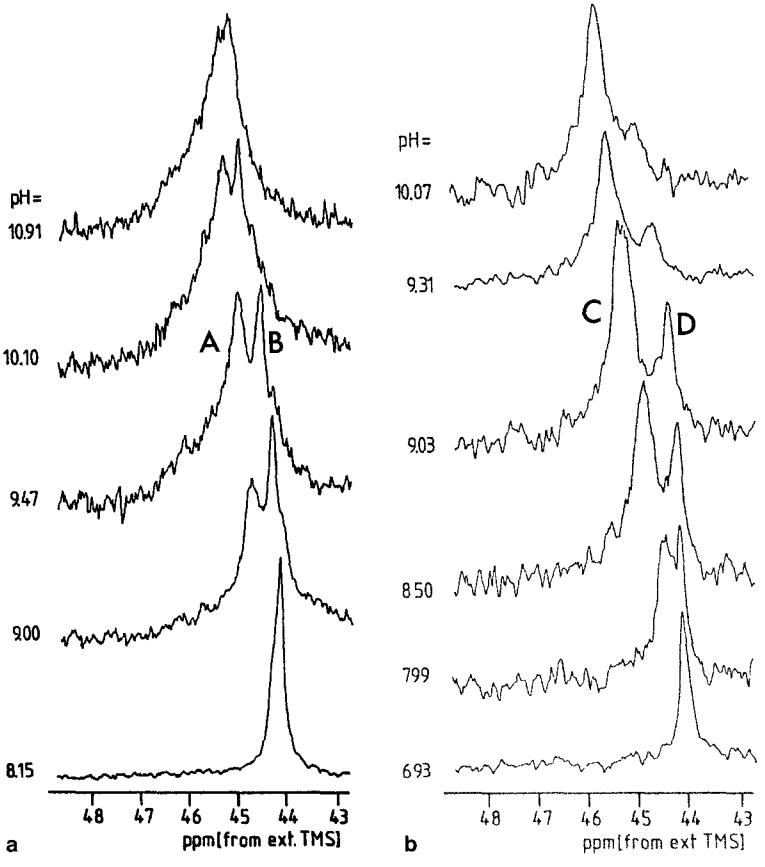

Fig. 1. 67.93 MHz ^{13}C-NMR spectra of free (**a**) and vesicle-bound (**b**) ^{13}C-labeled Nε-dimethyllysyl melittin at various pH values (10 m*M* melittin in 0.1 *M* sodium chloride, 0.1 *M* sodium borate). Protons were decoupled by using the WALTZ pulse sequence. For each spectrum, 200 scans at 25 °C were accumulated. The chemical shift was determined relative to the ^{13}C signal of tetramethylsilane (TMS) as an external reference

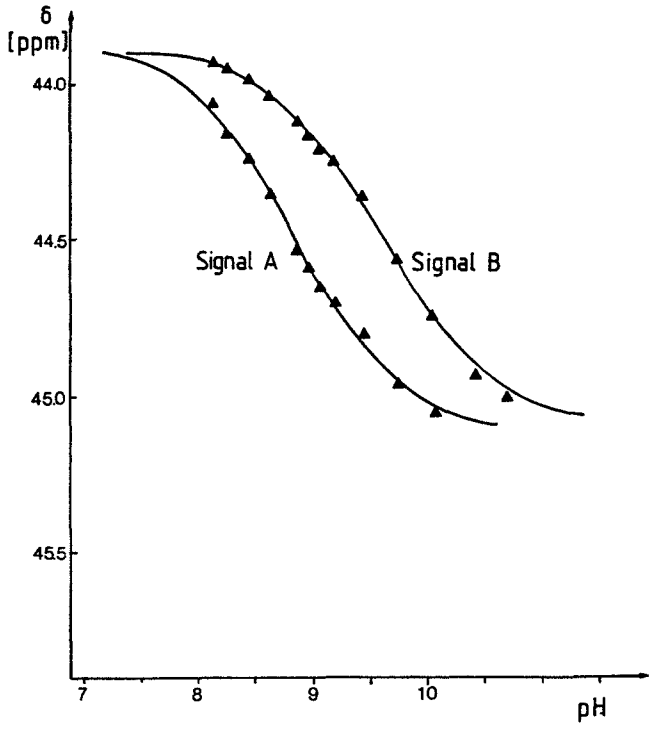

Fig. 2. pH dependence of the chemical shift of ^{13}C resonances of the Nε-methyl groups of modified, free melittin. The size of the triangles corresponds to the experimental error. Solid lines *A* and *B* represent titration curves evaluated by a fit program using the Henderson-Hasselbalch equation

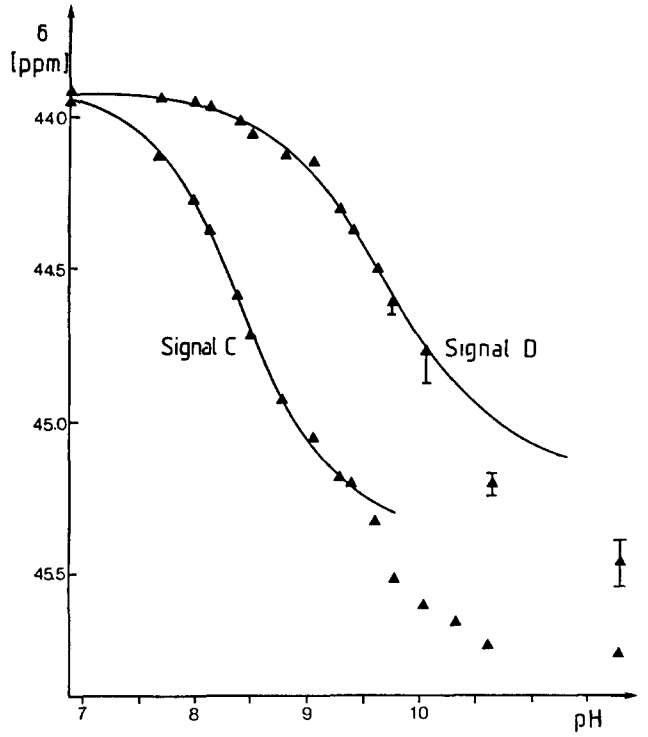

Fig. 3. pH dependence of the chemical shift of ^{13}C resonances of Nε-dimethyl-lysyl-melittin bound to lecithin vesicles. The fitted titration curves correspond to the signals *C* and *D* of the spectrum in Fig. 1 b. For the fit program only ^{13}C shift values between pH = 7 and pH = 10 were used

Table 1. pK values of the lysine Nε-dimethyl-amino groups of labeled melittin, calculated from the pH dependence of the chemical shift of the ^{13}C resonances of the adjacent methyl groups

	pK value	Hill coefficient	$\Delta\delta$ value[a] [ppm]
Tetrameric free melittin			
Signal *A*	8.84	0.92	1.1
Signal *B*	9.65	0.82	1.1
Vesicle-bound melittin			
Signal *C*	8.42	1.04	1.1 (1.6)
Signal *D*	9.64	0.88	1.1 (1.9)

[a] The given $\Delta\delta$ values were used for the calculation of the pK values according to the Henderson-Hasselbalch equation. The $\Delta\delta$ values in parentheses are the maximum values assumed to be dependent on a change of the chemical environment in addition to the pH dependence

the FIDs was applied in order to improve the resolution and to detect the chemical shifts at all pH values with higher precision. In Fig. 2 the pH dependence of the chemical shifts of the two ^{13}C resonances of the lysine amino methyl groups is plotted. The pK values, Hill coefficients, and differences of the chemical shifts, which were obtained using the Henderson-Hasselbalch equation with a computer fit program, are given in Table 1.

All NMR investigations on membrane-bound melittin were carried out at a molar ratio of lipid to protein of at least 100:1 in order to keep the lamellar structure of the liposomes intact. Fortunately, signals of the ^{13}C labels of melittin do not interfere with the natural abundance ^{13}C resonances of the lecithin (spectra not shown). Although the concentration of lecithin is higher than that of melittin by a factor of 100, the ^{13}C resonances of the melittin labels were of almost equal intensity. The pH dependence of the chemical shifts of the lysine ε-aminomethyl carbon resonances could be followed up to a pH value of 11.5. Above this pH value considerable hydrolysis of lecithin to fatty acids and lysolecithin was observed. As was also found for tetrameric melittin in aqueous solution, an increase of the pH value leads to a splitting of the ^{13}C resonances into one larger, broad signal *C* and one smaller, narrower signal *D* (Fig. 1 b). This splitting is even more pronounced than in the spectra of the free melittin (shown in Fig. 1 a). The titration curve derived from the pH dependence of the chemical shift values is plotted in Fig. 3. The values are different from those of free melittin and do not correspond to a one-step protonation-deprotonation equilibrium following the Henderson-Hasselbalch equation. With increasing pH a larger downfield shift of the resonances *C* and *D* (Fig. 3) as compared

to resonances *A* and *B* of free melittin (Fig. 2) was found. This observation may be explained with a change in the chemical environment of the amino group of the membrane-bond melittin at high pH values. It seems that interactions between the melittin side chains and the membrane lipids are responsible for this behaviour. The titration curves obtained for both resonances below pH = 9.6 seem to be normal and can be described by the Henderson-Hasselbalch equation, whereas above pH = 9.6 a pH-dependent change of conformation or orientation seems to occur, which leads to a second step in the titration curve and to an additional line broadening of resonance *D*. The pK values, Hill coefficients, and $\Delta\delta$ values derived from the fit of the titration curves below pH = 9.6 are given in Table 1. The pK values derived from the pH dependence of the resonance *D* and *B* of the membrane-bound and of the free melittin (Figs. 3 and 2) are almost identical, whereas the pK value obtained for the lysine derivative of membrane-bound melittin (resonance *C* in Fig. 3) is approx. 0.4 pH units lower than that of free melittin (resonance *A* in Fig. 2).

Relaxation time measurements

Spin-lattice relaxation times of the ^{13}C labels of free and membrane-bound melittin were determined together with the T_1 value of the choline N-methyl carbon resonance of lecithin. Decoupling of proton resonances was performed with the WALTZ pulse sequence. A logarithmic plot of the signal intensity versus the τ values of the inversion recovery pulse sequence yielded the T_1 values of Table 2. As can be seen from Table 2, T_1 of the ^{13}C labels drops by a factor of almost 2 when melittin is bound to the membrane. The relaxation time obtained for the carbon resonance of the choline methyl groups differs only slightly from those of the ^{13}C spin labels.

Table 2. T_1 values of the ^{13}C resonances of the melittin lysyl Nε-amino methyl groups ($T_{1,mel}$) and the lecithin N-methyl groups ($T_{1,PC}$) at 25 °C

Tetrameric free melittin	
at pH = 6.0	$T_{1,mel} = 0.98$ s
Vesicle-bound melittin	
at pH = 6.0	$T_{1,mel} = 0.50$ s
Melittin-containing lecithin vesicles	
at pH = 9.0	$T_{1,mel}$ (signal *D*) = 0.56 s
	$T_{1,mel}$ (signal *C*) = 0.48 s
	$T_{1,PC}$ = 0.40 s

Influence of shift reagents on the ^{13}C resonance of membrane-bound melittin

In order to obtain information about the orientation of melittin in phospholipid bilayers, the influence of shift reagents on the methyl carbon resonances of the methylated ε-amino groups of membrane-bound melittin as well as on the carbon resonances of the lecithin was investigated. It was necessary to select shift reagents which are water-soluble and presumably bind to the polypeptide or to the phospholipid in a specific way. Furthermore, shifts in resonance position should be induced through space, and the line width of the investigated resonances should not broaden significantly. Water-soluble shift reagents are chlorides and nitrates of certain lanthanides or their EDTA complexes. The lanthanide-EDTA complex has a negative surplus charge and therefore should interact with the positively charged lysine side chains or with the positive charge of the lecithin. The solvated, positively charged lanthanide ions should interact with the negative charge at the lecithin phosphate. In particular, the praseodymium cation induces a change in the chemical shift of an observed nucleus nearby, which is primarily dependent on the arrangement of the nucleus in space relative to the Pr^{3+} cation. The contribution to the chemical shift change, which is produced via the Fermi contact mechanism, amounts to only about 15% of the total induced change of the resonance position (Sievers 1973). Therefore praseodymium salts were chosen as shift reagents to investigate the ^{13}C shift changes of water-soluble melittin and membrane-bound melittin. In the presence of $PrEDTA^-$, a downfield shift of the methyl carbon resonances of methyl amino groups of melittin and lecithin was found. The shifts of the ^{13}C labels attached to the lysine residues were observed in free melittin as well as in membrane-bound melittin. However, the resonances of the two choline methylene groups were shifted even more downfield than the methyl resonances. Since the methylene groups are located near the negatively charged lipid phosphate group, it has to be assumed that competitive binding of Pr^{3+} ions to the phosphate group and to the chelating reagent occurs in addition to the binding of the praseodymium-EDTA complex to the positively charged groups of melittin and lecithin. Therefore an interpretation of the shift changes in the presence of $PrEDTA^-$ seemed difficult. On the other hand, Pr^{3+} ions are bound only to the phosphate group of the lecithin and no influence on the ^{13}C spectrum of free melittin was observed. On the addion of Pr^{3+} ions to vesicle suspensions, a drastic downfield shift of the ^{13}C resonances of the head group and the glycerol part of the outer lipid monolayer was detected (spectra

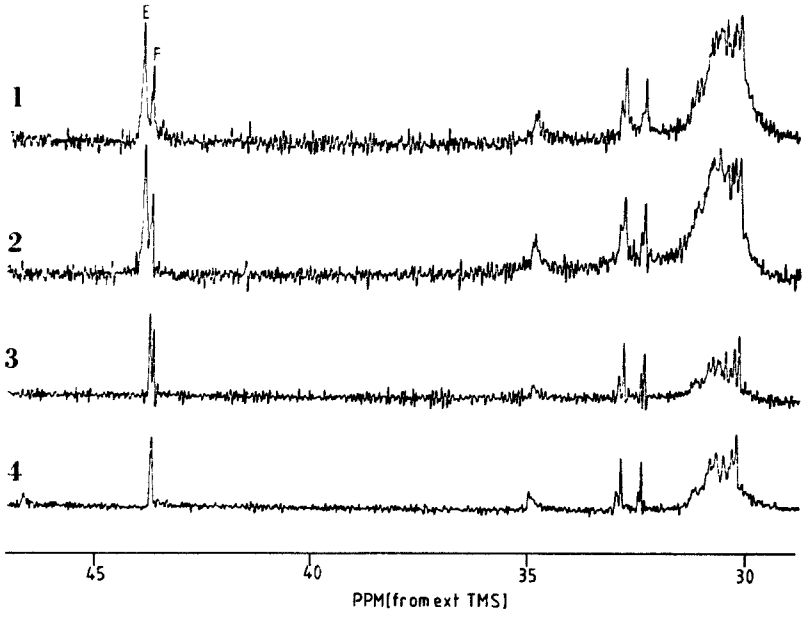

Fig. 4. 67.93 MHz ^{13}C spectra of vesicle-bound melittin at pH = 5.5 in the presence of various concentrations of Pr^{3+}. The $Pr(NO_3)_3$ concentrations are 60 m*M* (spectrum 1), 40 m*M* (spectrum 2), and 20 m*M* (spectrum 3), while the probe of spectrum 4 contained no Pr^{3+}. As in the spectra of pure vesicles, the ^{13}C resonances of the fatty acid residues are unaffected. The ^{13}C signals of melittin affected by Pr^{3+} in the outer medium of the vesicles are designated with *E* and *F*. Between 8,000 and 12,000 scans were accumulated for the spectra

not shown). Because of the close proximity to the phosphate group, the signals of the carbon resonances of the glycerol part and also of the C_1-methylene group of the choline part were affected most, whereas the carbon resonances of the fatty acid alkyl chains were not markedly influenced. The extent of the induced shifts decreases with the distances of the carbon atoms to the phosphate group. In addition, the shifts are linearly dependent on the Pr^{3+} concentration. At higher Pr^{3+} concentrations the signals were line-broadened.

The ^{13}C resonances of the vesicle-bound melittin are shifted downfield also upon the addition of Pr^{3+} (Fig. 4). In order to maintain the good solubility of the Pr^{3+} salt, a pH of 5.5 was chosen in this investigation. At this pH the ^{13}C resonances of the six methyl groups of the three lysine residues in melittin are represented by one single signal. Upon the addition of Pr^{3+} ions to the vesicle suspension, this resonance splits into two signals (*E* and *F* in Fig. 4). The smaller signal *F* is shifted only slightly, even at higher Pr^{3+} concentrations, whereas the signal *E* – of twice the intensity of *F* – is influenced to a larger extent. In Fig. 5 the induced chemical shifts of the carbon resonances of the choline carbons and of the methyl carbon labels of vesicle-bound melittin are plotted versus the Pr^{3+} ion concentration. Since it could be demonstrated that the ^{13}C labels attached to the ε-amino groups of the three lysines of membrane-bound melittin undergo a downfield shift in the presence of Pr^{3+} ions bound to the lipid phosphate groups, it should be possible to roughly determine the distance between the lysine amino groups and the membrane surface from the extent of the induced chemical shift changes. In addition, the

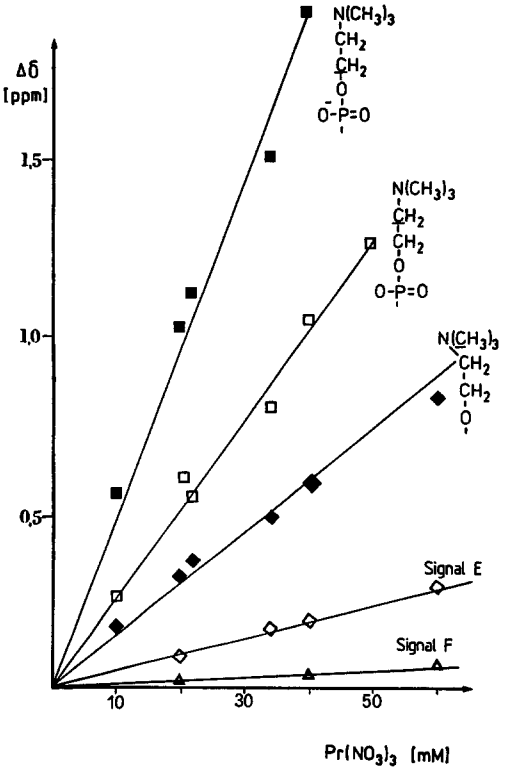

Fig. 5. Pr^{3+}-induced chemical shifts of the ^{13}C resonances of lecithin and of vesicle-bound ^{13}C-labeled melittin plotted versus the concentration of Pr^{3+} in the outer medium of the vesicles. The resonances of modified melittin are designated with *E* and *F*, as in spectrum 1 of Fig. 4

induced chemical shift changes should reveal whether lysine side chains may penetrate from the outside of the membrane across the bilayer into the inside of the vesicle. A prerequisite for this investigation is that only either the outer or the inner bilayer is accessible to the shift reagent, or that Pr^{3+}

ions can bind only at either the outer or the inner lipid monolayer of the vesicles. It has to be proven that the vesicle preparations stay intact in the presence of the potentially membranolytic melittin, so that the Pr^{3+} ions cannot cross the membrane barrier. Two possible processes have to be considered: Melittin could form within the bilayer membrane a pore that allows Pr^{3+} ions to pass. Or, owing to trace amounts of phospholipase A_2, a lysis of the phospholipid could occur. Both possibilities for the penetration of Pr^{3+} ions across the membrane were excluded by observing the two carbon resonances of the lecithin N-methyl groups. In case of an enhanced diffusion of Pr^{3+} ions across the membrane, a decrease of the difference in chemical shifts between the signals of the inner and of the outer lipid monolayer should be observed. Penetration of Pr^{3+} ions into the inner vesicle space could indeed be followed by observing the ^{31}P resonances of the lecithin phosphate groups in the presence of melittin containing phospholipase A_2 as an impurity (spectra not shown). Not even after 48 h did phospholipase A_2-free melittin lead to a coalescence of ^{13}C resonances or ^{31}P resonances of the lecithin. Likewise, the intensities of the choline methyl carbon resonances remained unchanged in the presence of melittin.

Three types of vesicle suspensions were prepared. One suspension contained Pr^{3+} ions in the outer medium partially bound to the external lipid monolayer; the second contained the paramagnetic ions in the inner and outer medium in equal concentrations, and the third contained Pr^{3+} only in the inner volume of the vesicles. In all preparations the concentration of lecithin (50 m*M*), melittin (0.5 m*M*), and $Pr(NO_3)_3$ (50 m*M*) were the same. In order to reach a good spectral resolution, a Gaussian filtering of the FIDs was applied. Since for this procedure the signal-to-noise ratio decreases and because of the low concentrations of melittin, acceptable spectra were obtained only after 12 to 14 h. In Fig. 6, ^{13}C spectra of vesicles containing Pr^{3+} ions in the inner volume with (spectrum 1), and without melittin (spectrum 2) are compared with a melittin-containing preparation without the shift reagent (spectrum 3). When Pr^{3+} is included in the inner volume, the chemical shift of the resonances of the melittin-^{13}C label is identical with that of the Pr^{3+}-free vesicle preparation (spectra not shown), whereas the ^{13}C resonances of the choline methyl groups of the inner lecithin monolayer are shifted by approx. 0.8 ppm to lower field in the presence of Pr^{3+} ions. From the induced change of the chemical shifts of the lecithin-choline resonances it could be concluded that the effective concentration of Pr^{3+} ions in the inner volume of the vesicles is equal to the concentration of Pr^{3+} in the outer volume of the vesicle suspension of spectrum 2 (Fig. 6).

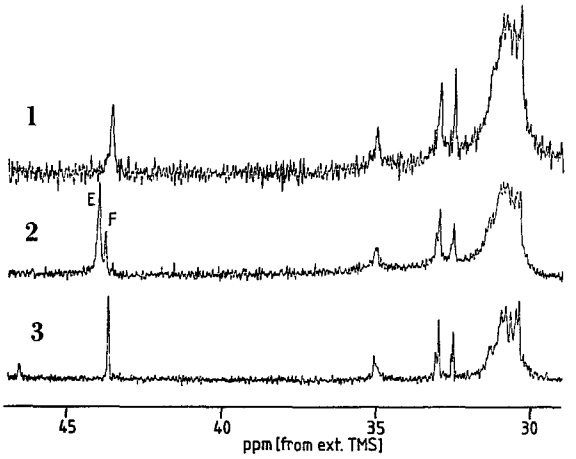

Fig. 6. 67.93 MHz ^{13}C spectra of vesicle-bound ^{13}C-labeled melittin at pH = 5.5. While spectrum 3 was obtained from a probe without Pr^{3+}, the vesicles of the other probes contained Pr^{3+} in the outer medium (spectrum 2) or only in their internal volume (spectrum 1)

Apparently Pr^{3+} ions induce shifts in resonance position of the melittin carbon resonances only when the cation and melittin are located on the same side of the membrane.

Discussion

Although quite a number of investigations have been carried out on the structure and conformation of water-soluble melittin, the orientation of membrane-bound melittin is still uncertain. Four main models of the orientation of melittin inside a bilayer membrane are discussed. In all models it is assumed that the N-terminal hydrophobic area is α-helical and forms an angle of about 120° with the rest of the molecule at Pro 14. An additional common feature of all models is that the C-terminal basic amino acid residues 21 to 24 remain accessible to the solvent at the side from which the polypeptide penetrates the membrane. The four models of arrangement of melittin in the bilayer membrane are shown in Fig. 7. In model *A*, which was derived from neutron scattering experiments (Strom et al. 1983), the N-terminal part of melittin is assumed to cross the bilayer membrane in such a fashion that on both sides of the membrane the charges of the polypeptide are accessible to the solvent. Tosteson and Tosteson (1981) described the membrane-bound melittin as a tetramer of identical subunits (model *B*) with essentially the same conformation as in model *A*. However, the sides of the helices having a more hydrophilic character should be oriented

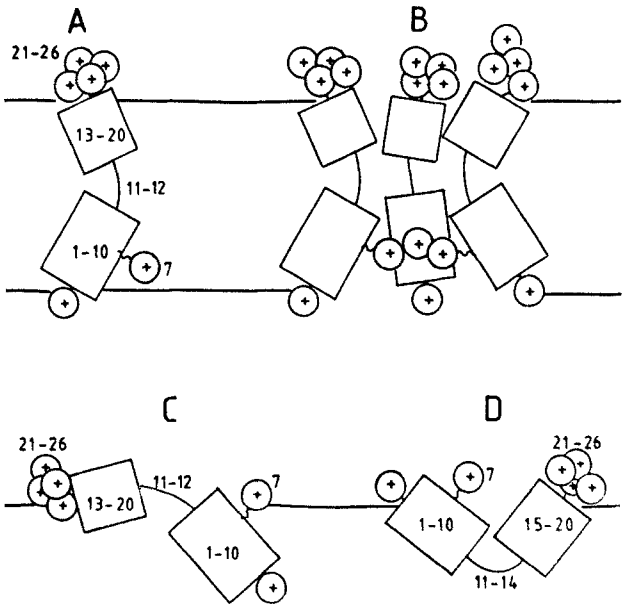

Fig. 7. Schematic representation of the current models for the orientation of melittin in phospholipid bilayers. In model *A*, melittin is a membrane-spanning monomer; a tetrameric pore complex of these monomers is formed in model *B* (for the sake of clarity the fourth monomer is not shown). According to model *C*, monomeric melittin is bound to the periphery of the membrane, whereas in model *D* it is partially inserted and takes on a wedge-like conformation. The amino acid residues are indicated by their sequence position. The α-helical segments of melittin are shown as rectangles, the peptide backbone, as a solid line. Although the conformation of the C-terminal part 21 to 26 is α-helical according to X-ray analysis, no defined conformation is ascertained for this sequence

towards each other, whereas the more hydrophobic areas should have contact with the acyl chains of the phospholipids. Based on an X-ray structure analysis, Terwilliger et al. (1982) discussed a conformation in which monomeric melittin is bound at the surface of the bilayer membrane in such a way that the axis of the helix between residues 13 and 26 is oriented parallel to the membrane surface. In this model (model *C*) the N-terminal part with at least one charge should partially penetrate into the membrane. Finally, a conformation (model *D*) has been proposed (Dawson et al. 1978), in which both helices penetrate partially into the outer monolayer, whereas the N-terminal and the C-terminal parts are still accessible to the aqueous phase. In view of these models the NMR parameters of ^{13}C-enriched methyl groups introduced at the ε-amino groups of the lysines in melittin shall be discussed. The results are expected to lead to statements about possible electrostatic interactions and about flexibility and accessibility of the lysine chains of membrane-bound melittin, which may or may not be in agreement with the models of the melittin-membrane complex.

At low pH values a single resonance for the ^{13}C-labeled methyl groups was observed in the NMR spectra of water-soluble tetrameric melittin. With increasing pH this single resonance was split into one smaller resonance *A* and one larger resonance *B*. In addition, a relatively weak signal, shifted slightly more downfield, appeared in the same absorption region. All three methyl carbon resonances were pH-dependent. From the pH dependence of the chemical shifts titration curves were obtained from which pK values were calculated using the Henderson-Hasselbalch equation. A pK value of 9.65 was assigned to the resonance *A*, whereas a pK value of 8.84 was obtained from the pH dependence of resonance *D*. The weak signal was correlated with a pK value of 6.8. Looking at the amino acid sequence of melittin makes an identification of resonances and pK values relatively easy. Since with a reductive methylation the N-terminal α-amino group was only slightly affected and since this group usually has a pK value around 7, the weak signal may be assigned to the methyl group attached to the N-terminal α-amino group of glycine 1. Besides the positively charged α-amino group only one additional positively charged side chain, namely lysine 7, is located in the sequence 1 to 20. Since the protonation equilibrium of the ε-amino group of lysine 7 should not be influenced by interionic interaction with other side chains, one would expect for this side chain a normal titration curve and a pK value almost identical with that of a dimethylated lysine Nε-amino group. This pK value is approx. 0.6 pH units lower than that of unmethylated lysine side chains in proteins (Brown and Bradbury 1975). Therefore lysine 7 in native melittin should have an almost normal pK value of about 10.2, as would be expected for a lysine side chain that is freely accessible to the solvent water. Lysines 21 and 23 together with the arginine residues 22 and 24 form a cluster of positive charges. The repulsive forces of these charges may lead to smaller pK values of the lysine residues 21 and 23 in agreement with previous results (Quay and Tronson 1983). Therefore the larger signal *B* in Fig. 1 a was assigned to the carbon resonances of the ε-amino methyl groups of lysine 21 and 23. In the spectra of vesicle-bound ^{13}C-methyl melittin two signals with different intensities and different pH dependence of chemical shifts were observed. The titration curve determined from the pH dependence of the smaller resonance *D* of Fig. 1 b was almost identical with the titration curve of lysine 7 in free melittin (signal *B* in Fig. 1 a). The pK value derived therefrom is identical with that of

lysine 7 in the free polypeptide. Hence, resonance *D* was also assigned to lysine 7 in the membrane-bound melittin. A pK value of 8.42 was obtained from the titration curve determined from the pH dependence of resonance *C* in Fig. 3. Assuming that this signal – twice as intense as signal *D* – can again be assigned to the two lysines 21 and 23, the pK values of the two lysines in the membrane-bound melittin are considerably lower than those of the free melittin. Since an interaction of the dimethyl ammonium groups with the negatively charged lipid phosphate would lead to an increase in the pK values, it has to be assumed that, owing to the rigidity of the membrane surface and to a restricted flexibility of the side chains, the positive charges of the cluster may be brought even closer together, which will lead to a further decrease in pK. It is also possible that the positive charge at the choline amino group may be responsible for the decrease in pK. From the shape of the titration curve of lysines 21 and 23 it can be concluded that once the ε-amino groups are deprotonated a rearrangement of the side chains above pH = 9.6 occurs. Possibly, above pH = 9.6 the lysine side chains are inserted between the acyl side chains of the lecithin.

The line widths of the investigated methyl carbon resonances of the lysines in membrane-bound melittin did not change during the course of the titration. It seems that there is a quick exchange between the protonated and the deprotonated amino groups. Apparently the lysine side chains are oriented into the solvent and are freely accessible to the titrant. In order to make sure whether the observed signals *C* and *D* of Figs. 1b and 3, which were assigned to lysines 21 and 23 or to lysine 7, both originate from the membrane-bound melittin, spin-lattice relaxation times (T_1) of these ^{13}C-labeled methyl groups were determined. In free tetrameric melittin a T_1 value of 0.98 s was found for the methyl groups of the protonated lysine side chains. For vesicle-bound melittin at the same pH, the T_1 value was 0.5 s. At a pH of 9.0 the relaxation time for lysine 7 was found to be 0.56 s and for lysine 21 and 23, 0.48 s. In the same experiment, T_1 of the choline N-methyl groups of lecithin was determined to be 0.4 s. The drop in the T_1 values for melittin in the presence of vesicles indicates that melittin indeed is bound to the membrane. Of the three lysine side chains the one of residue 7 seems to be more flexible than those of lysine 21 and 23. However, the T_1 values of the methyl groups of lysines 21 and 23 are larger than those of the methyl groups of the lipid head groups. Therefore a location of the lysine 21 and 23 side chains in the neighbourhood of the glycerol part of lecithin is unlikely, for this molecular part is the most rigid of the whole lipid molecule. A drastic immobilisation of the lysine side chains was not observed. Therefore a direct ionic interaction with the lecithin phosphate groups does not seem to occur.

In order to further investigate the orientation of melittin in a bilayer membrane, the shift reagent praseodymium nitrate was added to the solution. Praseodymium ions induce shifts in resonance position which are not mediated via the bond electrons but rather through space. These shift changes can be described by the McConnell-Robertson equation. In this equation an axial symmetry of the magnetic dipole field and the lanthanide substrate complex is assumed, with the axis of the dipole oriented to the lanthanide substrate bond in a colinear way. The shift in resonance position of the NMR-sensitive nuclei of the substrate is dependent on the distance *A* from the paramagnetic centre and on the angle between the vector *A* and the direction of the lanthanide substrate bond. In addition, the induced change of the chemical shift is proportional to the mole fraction of the lanthanide substrate complex. Assuming that the Pr^{3+} ions can bind only to the negatively charged phosphate groups of the lipids, a rough estimate of the distances of the investigated nuclei to the paramagnetic centre seems possible from the shift changes. At a constant Pr^{3+} concentration the $\Delta\delta$ values of the choline carbon resonances of lecithin decrease from C_1 to C_2 and further to the N-methyl groups. Unfortunately, the signals of the glycerol carbon resonances were broadened to such an extent that a precise determination of $\Delta\delta$ was not possible. According to the observed shifts, the glycerol part of lecithin seems to be oriented parallel to the dipole axis.

The choline part of the lecithin is less defined in structure. As already mentioned, considerable $\Delta\delta$ values for the ^{13}C resonances of the choline N-methyl groups were observed, although they are assumed to be localized further away from the Pr^{3+} binding site.

In the presence of Pr^{3+} ions the signal of the ^{13}C resonances of the lysine methyl groups of membrane-bound melittin is split into one smaller signal (*F* in Fig. 4) and one signal of double intensity (*E* in Fig. 4). With increasing concentration of $Pr(NO_3)_3$ the resonance *E* was shifted further downfield. The $\Delta\delta$ value amounts to about half that of the lecithin N-methyl groups. The signal *E* corresponds to the ^{13}C labels of two lysine residues, both of which could have a similar distance from the Pr^{3+} binding site in the time average. Since the shift reagent is not bound to the melittin molecule but rather to the membrane lipid, the orientation of the lysine methyl groups relative to the Pr^{3+} substrate bond is undetermined and the distance to the paramagnetic centre can not be derived. For the signal *F* in Fig. 4,

a small but definite shift in resonance position was observed upon the addition of Pr^{3+} ions. Therefore this resonance should be assigned to a lysine residue that is located further away from the paramagnetic centre. It seems unlikely that one of the two lysines 21 and 23 is located further away from the lecithin phosphate group, while the other lysine residue is at the same distance from the Pr^{3+} site as lysine 7. Therefore we would rather assign the signal *F* to the methyl carbon resonance of lysine 7, whereas signal *E* should be assigned to the lysine residues 21 and 23. Consequently, lysine 7 maintains a position further away from the membrane lipids. This arrangement corresponds to the fact that the pK value of the lysine-7 amino group does not change with the melittin membrane complex formation. Lysine residues 21 and 23 are more affected by the paramagnetic centre. The induced shifts amount to approximately half of the $\Delta\delta$ value observed for the choline N-methyl groups. It seems that the lysine residues 21 and 23 are both positioned near the positively charged choline amino groups of lecithin. This proximity would also explain the lower pK values, compared to free melittin. It should be emphasized, however, that the side chains are oriented towards the membrane surface and that they are freely accessible to the solvent water.

Having localized the ^{13}C labels of melittin relative to the membrane surface, we can describe the orientation of the polypeptide in the membrane. If the hydrophobic amino acid sequence 1 to 20 of melittin spanned the bilayer membrane, the ε-amino group of lysine 7 should appear in the neighbourhood of the inner monolayer phosphate groups. From the Pr^{3+}-induced shift in resonance position of the lysine-7 methyl groups it follows, however, that this side chain does not maintain such a position. Therefore a membrane-spanning conformation of the peptide can be excluded. It was further shown that the shift reagent induces a chemical shift change only when it is added to that side of the membrane from which melittin penetrates into the vesicles, i.e., the outer medium of the vesicles. Since Pr^{3+} ions included in the inner volume of the vesicles do not have an influence on any of the three lysine residues, it can be concluded that the lysine side chains are located on the outer surface of the vesicles. It is possible that the introduction of two methyl groups at each of the three α-amino groups prevents penetration of the charged side chains into the membrane. However, in the course of our calorimetric investigation of the melittin-lipid interaction (unpublished results) we have demonstrated that the ΔH values for the interactions of phospholipid vesicles with native melittin and with the modified melittin are identical.

The results discussed so far contradict models *A* and *B* in Fig. 7 for the orientation of melittin in membranes. Therefore some of the previous studies are reviewed and compared with the investigations described in this paper.

Tosteson and Tosteson (1981, 1984) determined the conductivity of melittin-containing lecithin membranes. They found an increase of permeability with respect to anions, which was dependent on the fourth power of the melittin concentration. From this result the existence of a tetrameric pore complex of melittin was derived. Tetrameric melittin was assumed to be in equilibrium with monomeric, membrane-spanning integral melittin (model *B* in Fig. 7). Commercial melittin without further purification was used for the experiments. Also, no chelating reagent such as EDTA was added to bind the Ca^{2+} ions and thus to inhibit traces of phospholipase A_2. In fact, phospholipase A_2 impurities may have induced the permeability of the membranes for anions. From conductivity measurements with melittin-containing phospholipid membranes, Schoch and Sargent (1980) did not derive evidence for the appearance of the N-terminal part of melittin on the other side of the membrane. In similar experiments, Kempf et al. (1982) determined the voltage-dependent conductivity of melittin-containing black lipid membranes. By observing the enzymatic proteolysis of melittin on either the *cis*- or the *trans*-side of the membrane, they could show that a penetration of the N-terminal part of melittin across the membrane is possible only in the presence of a membrane potential. A voltage-dependent pore formation was observed by other groups (Hanke et al. 1983). Extensive ^1H and ^{13}C investigations were carried out by Podo et al. (1982), who studied in particular ^1H line width changes of the melittin amino acid side chains upon addition of small amounts of phospholipids. Although a model for the orientation of melittin in the membrane was derived, the data is restricted to the interaction of melittin with lipid micelles. The orientation of melittin with respect to membranes was investigated by using polarized IR-spectroscopy (Vogel et al. 1983). According to these studies the axes of both melittin helices form an angle of approx. 45° with the membrane normal. This result can be interpreted with model *B* of Fig. 7 as well as with a wedge-like orientation (model *D* in Fig. 7).

The formation of tetrameric melittin in membranes leading to pores cannot be excluded at high melittin concentrations. Occasionally during our ^{13}C-NMR experiments, because the protein was not thoroughly dispersed in the solution, local high melittin concentrations led to a leakage of part of the vesicles. In addition, the penetration of melittin

through the lipid bilayer membrane may be catalysed by hydrophobic anions, i.e., fatty acid impurities. Apart from this experimental artifact, there is no evidence that spontaneously bound melittin in low concentrations forms an integral part of the bilayer membrane according to models *A* and *B*.

With respect to the secondary structure of membrane-bound melittin, the ^{13}C-NMR investigations have to be discussed in view of the data of previous results. Terwilliger et al. (1982) assumed that the secondary structure of melittin bound to the periphery of membranes is the same as that obtained for crystalline melittin. In the crystal, melittin maintains an α-helical structure that is formed throughout the sequence with the exception of a bend near the proline-14 residue, whereas from other investigations it was suggested that the extent of helix formation is only 75%, i.e., about 20 amino acid residues are involved in the α-helix formation. In the presence of lipid micelles the helix content was found to be slightly reduced. Using 2D-^{1}H-NMR techniques, Brown et al. (1982) investigated melittin which was bound to micelles formed by deuterated dodecylphosphocholine. Distances between various protons were obtained from NOE effects, which again were used to calculate the secondary and tertiary structures of the polypeptide with a distance geometry algorithm. According to this work, between residues 16 to 20 a right-handed helix is formed with an axis oriented at an angle of 60° and 90° relative to the micelle surface. The secondary structure of the N-terminal part of melittin was not described. Also from 2D-^{1}H-NMR measurements of tetrameric melittin dissolved in water, an α-helix between residues 16 to 20 was derived, whereas for the N-terminal part and the C-terminal region 21 to 26 no definite conformation could be determined. More indirect evidence for a disordered structure of the C-terminal part was obtained from investigations of the interaction of antibodies with melittin. King et al. (1984) observed that the C-terminal sequence 20 to 26 serves as a recognition region for the melittin-specific antigen-antibody interaction. In general, the recognition sites for antibodies in proteins possess high flexibility and a more random structure. In fact, the four basic amino acids 21 to 24 may be compared to polylysine, which does not maintain a secondary structure at neutral pH values. Therefore it seems reasonable that the C-terminal part of melittin in solution does not form an α-helix. In the membrane-bound state of melittin the formation of an α-helix in the C-terminal part should occur only when the positive charges of the lysine or arginine side chains are compensated by interactions with lipid phosphate groups. However, this interaction should lead to an increase of the pK values of lysines 21 and 23 with respect to the free melittin. This increase in pK was not found in our ^{13}C-NMR investigations. Therefore, contrary to the model of Terwilliger, it has to be assumed that the C-terminal basic amino acid residues are located in a similar distance to the choline methyl groups and retain high flexibility.

The α-helix of crystalline melittin has a kink between residues threonine 11 and glycine 12. This interruption is induced by essentially two amino acid residues. Proline 14 cannot form a hydrogen bond to threonine 10, since the imino nitrogen is part of the pyrrolidine ring system. Torsions around the N−C bond are not possible and the dihedral angle of 15°, which is optimal for an α-helix, cannot form. Since glycine 12 does not contain a bulky side chain, the kink in the helix induced at the proline 14 residue is facilitated. But also the formation of a β-turn between residues 10 to 14 seems possible. In this case the angle between the two helix axes would be smaller than 120° and the polypeptide would have a wedge-like structure. Because of the β-turn the N-terminal helix would turn around its axis by about 180°. The side chain of lysine 7 would maintain a position between the two helix parts and would be oriented towards the aqueous solution. In ^{1}H-NMR investigations of melittin in the presence of micelles no NOE effect between the C-terminal and the N-terminal helix was found (Brown and Wüthrich 1981). This seems to be consistent with an interruption of the α-helical conformation by a β-turn. In addition, the angle of 45° of the two helices relative to the membrane normal, which was derived by Vogel et al. (1983), is further evidence for a wedge-like conformation of the polypeptide. Brown et al. (1982) studied the line-broadening of melittin proton resonances which were induced by micelle-bound fatty acids containing paramagnetic doxyl derivatives in different positions of the hydrocarbon chains. They concluded from this investigation that the penetration depth of the polypeptide in the micelle is of the order of 0.8 to 1 nm. Although the micelle system is not comparable to the lecithin vesicles, the results with respect to the position of the three lysine side chains of melittin are in agreement with the results obtained from the ^{13}C-NMR investigation. A wedge-like conformation is certainly a realistic model for the membrane-bound melittin, which is in agreement with most of the data published in the literature.

The study of the orientation of melittin in neutral lipid membranes indicates the dominating influence of charges preventing a penetration of the polypeptide into the membrane. Although lysine 7 is part of an α-helical sequence of 20 mainly hydrophobic side chains, which is long enough to span the

membrane, this helix is not inserted, nor does the side chain appear on the opposite side of the bilayer. Apparently the positive charges act as an anchor to keep the protein at one side of the membrane.

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