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# The Aggregation State of Spin-Labeled Melittin in Solution and Bound to Phospholipid Membranes: Evidence That Membrane-Bound Melittin Is Monomeric

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*ABSTRACT* **Spin-labeled derivatives of the bee venom protein, melittin, were obtained by reacting on the average one of the four amino groups of the protein with succinimidyl-2,2,5,5-tetramethyl-3-pyrroline-1-oxyl-3-carboxylate. All 16 statistically possible reaction products with 0, 1, 2, 3, or 4 spin labels per protein were then separated in a single pass with reversed phase high performance liquid chromatography. With the help of trypsin digestion and diode array detection it was possible to assign the primary structure of all 16 eluting fractions. All fractions with only one spin label per protein were purified for electron paramagnetic resonance measurements. The labeling sites cover different regions of the protein: one is at the N-terminus, one at lysine-7, and two are near the C-terminus at lysine-21 and lysine-23, respectively. This set of specifically labeled melittins was used to study the structure and dynamics of melittin in aqueous solutions and when bound to neutral or negatively charged membranes. In aqueous solution a reduction in rotational correlation time and appearance of spin-spin interaction was observed during salt-induced transition from a random coil monomer to a mostly $\alpha$-helical tetramer. Membrane binding to phospholipid bilayers in low or high ionic strength was reflected only in a further decrease in mobility. The absence of any spin interaction in the membrane-bound state suggests that melittin is monomeric under these conditions. All derivatives were able to detect these structural changes, but melittin labeled at the N-terminal amino group was especially valuable. Because of postulated intramolecular hydrogen bonding, this label reflects directly the motion of the entire protein or tetramer. Broadening experiments with chromium oxalate show that all labeled sites are at least partially exposed to the aqueous phase when melittin is bound to membranes. This suggests that an $\alpha$-helical melittin monomer binds to membranes with its axis parallel to the membrane surface.**

Key words: melittin, spin-labelling, EPR spectroscopy, membrane-protein interaction, protein-protein interaction

## INTRODUCTION

Melittin is a water-soluble 26 amino acid polypeptide, and one of the major constituents of bee venom.[3] In solution at high ionic strength, it forms tetramers stabilized by hydrophobic interactions between nonpolar faces of four amphiphilic helices. At low ionic strength, electrostatic repulsions between the monomers destabilize the tetramer and the polypeptide adopts a random coil structure.[36]

It has been reported that voltage dependent conductances are induced in membrane systems in the presence of melittin.[15,45] Indeed, single-channel activity has been observed under patch clamp conditions. While there is some debate as to the mechanism of the induced ion conductance, there is little doubt that the protein binds strongly to phospholipid bilayers and native membranes and causes changes in the membrane permeability. Thus, melittin is an intriguing model for membrane-protein interaction, and the structure of the membrane-bound form is of significant interest.

While the melittin tetramer has been crystalized[40,41] and is known to high resolution, the structure of the membrane-bound state is much less certain. It is generally recognized that the secondary structure of the membrane-bound form is highly $\alpha$-helical, but the degree of aggregation, tertiary structure, and orientation of the individual helices are a matter of debate.[16,38,47]

The present paper describes the synthesis and characterization of a number of spin-labeled derivatives of melittin and their application to the study of the structure of the polypeptide bound to phospholipid bilayers. The electron paramagnetic resonance (EPR) spectra of spin-labeled melittin provide detailed information on local conformation and environment. With several different labeling sites, sufficient information can be obtained to allow conclusions regarding global conformation and structure of this small peptide in a membrane system.

Covalently attached spin labels are powerful tools to obtain structural and motional information of proteins with EPR spectroscopy. They can also be used in long-known, but recently improved nuclear magnetic resonance (NMR) methods to determine 3-D

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structure in solution.[26] To get specific information, it is essential to attach the spin label only to one known site of the protein, and this is the main challenge. Ideally, one wishes to be able to attach a single spin label very specifically to any desired position in the protein. One way to achieve that is by incorporating into the protein by peptide synthesis a reactive site where a spin label can be attached. Under favorable conditions it is also possible to replace any amino acid with a uniquely reactive one that is not endogenous to that protein (e.g., cysteine) by site-directed mutagenesis. To this engineered site one can then attach a spin label.[44] These techniques offer, of course, the highest flexibility and make every site on the protein available for study.

Progress in high resolution separation techniques have made it also possible to randomly react endogenous reactive side chains with a spin label, followed by preparative separation of all statistically possible reaction products.[2] While this method cannot provide a spin label at any desired position, it can yield a sufficient number of different labeled sites to cover the most interesting portions of the protein. This article describes the application of this approach to spin-label the bee venom protein, melittin.

A common problem of these methods is the small amount of spin-labeled material usually available. Fortunately, the sample requirements for EPR measurements have been significantly reduced by the introduction of the loop-gap resonator to EPR spectroscopy.[13] This technology permits measurements of spin-labeled proteins in the picomole range and, in combination with the above mentioned spin labeling techniques, opens new areas of biological research to EPR spectroscopy. As an added bonus loop-gap resonators also facilitate and improve more sophisticated EPR applications like dispersion mode EPR,[22] ELDOR spectroscopy,[21,23] saturation transfer EPR,[43] rapid mixing kinetic studies,[19] and saturation recovery EPR in a biological system.[1,10] Recent progress made in all the above-mentioned fields is expected to dramatically increase the value and importance of spin labeling studies in biological systems.

## MATERIALS AND METHODS

### Solvents and Chemicals

Melittin was obtained from Sigma (St. Louis, MO) and succinimidyl-2,2,5,5-tetramethyl-3-pyrroline-1-oxyl-3-carboxylate from Molecular Probes (Eugene, OR). Water was purified with a Milli-Q system (Millipore Corporation, Bedford, MA), had a resistance of 18 MΩ, and was free of any detectable organic impurities. Acetonitrile from Burdick & Jackson (Muskegon. MI), and phosphoric acid (85%) and sodium perchlorate from Fischer (Pittsburgh. PA) were all HPLC grade. Boric acid and acetic acid were reagent grade. Trypsin treated with TPCK to inhibit chymotrypsin activity was obtained from Worthington Biochemical Corporation (Freehold, NJ). Dioleoylphosphatidylcholine (DOPC) and palmitoyl-oleoyphosphadidic acid (POPA) were obtained from Avanti Polar Lipids, Inc. (Birmingham, AL) and used without further purification. Chromium oxalate (potassium trioxalatochromiate) was obtained from ICN-Pharmaceuticals (Plainview, NY). Outdated red blood cells were obtained from the blood bank of the UCLA Medical Center in Los Angeles.

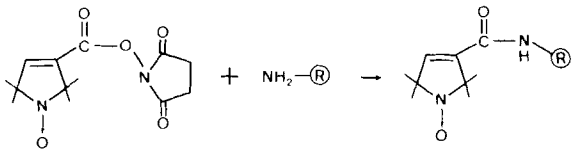
### EPR Measurements

A Varian E-109 EPR spectrometer (Varian Associates Inc., Palo Alto, CA) operating at X-band and fitted with a two-loop-one-gap resonator and interfaced to a NICOLET 1280 computer (Nicolet Instrument Corporation, Madison, WI) was used in all EPR experiments. To provide a stable AFC lock with the loop-gap resonator, the microwave bridge was modified, as described elsewhere.[19] The active sample volume in the loop-gap resonator is approximately 1 μl. Because of the difficulties involved in handling such small volumes with high precision, 4–10-μl samples were usually prepared. These samples contained on the average 1 mg/ml spin-labeled melittin. The average total amount per experiment was therefore < 1 nmol melittin. However, note that the amount actually needed for the resonator is only 350 pmol. The actual concentrations of melittin, buffer, salts, and phospholipids are given in the figure legends.

Rotational correlation times were obtained by matching the EPR spectra to a computer-simulated library of spectra. The computer simulations were done using an improved version of the Fortran program developed by Freed and co-workers[12] running on a VAX 11/780 computer equipped with a floating point accelerator.

### Synthesis of Spin-Labeled Mellitin Derivatives

The spin label succinimidyl-2,2,5,5-tetramethyl-3-pyrroline-1-oxyl-carboxylate acylates amino groups (e.g., lysine side chain or N-terminus of a protein) according to the scheme shown below.




The reaction was carried out at room temperature in a 1:1 mixture of 50 mM boric acid buffer, pH 10, and acetonitrile in a total volume of 1 ml. The final concentration of melittin in the reaction was 30 mg/ml. The spin label was added as a concentrated stock solution in acetonitrile. Equimolar amounts of spin label and protein were used to obtain a statistical mixture of all possible reaction products with 0, 1, 2, 3, and 4 spin labels per melittin. The spin label was

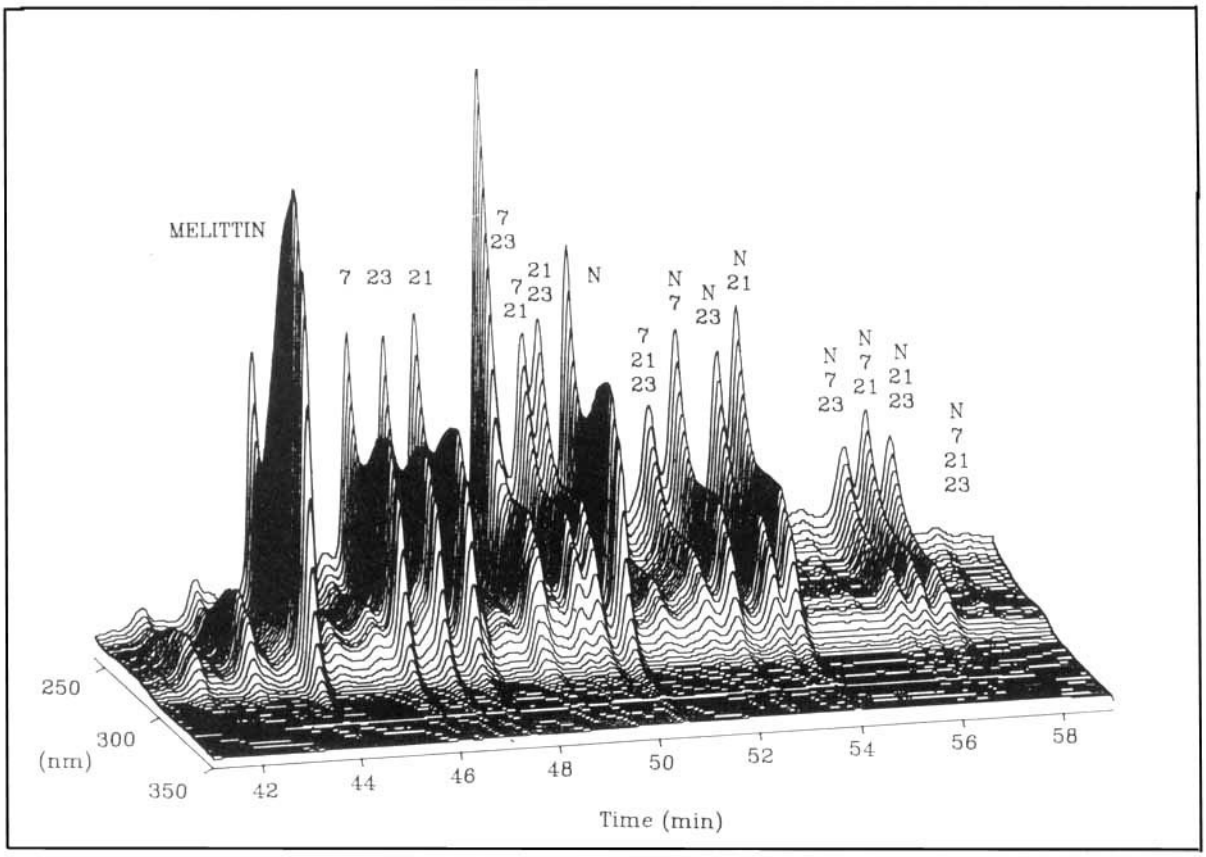



Fig. 1. Three-dimensional chromatogram of the preparative separation of the products of the reaction of melittin with the spin label succinimidyl-2,2,5,5-tetramethyl-3-pyrroline-1-oxyl-3-carboxylate as obtained with the diode array detector. To the right is the time axis and to the left the wavelength axis. The Z-direction indicates optical density. All separation conditions are given in the text. Because there are four potential attachment sites for the spin label, there are 16 different melittin products possible: unreacted melittin, four different isomers with one label attached, six isomers with two labels attached, four with three labels, and one with all four amino groups reacted. The number above each peak indicates the residue numbers to which the labels are attached. N means the label is attached to the N-terminus.

added slowly and with stirring to avoid local high spin-label concentrations leading to large relative amounts of unlabeled and tetralabeled melittin. Under these conditions all amino groups are fully deprotonated and react with equal probability with the spin label. There are no detectable side reactions, and the reaction is complete in less than 5 minutes as judged by HPLC analysis of the reaction mixture.

### Separation of Spin-Labeled Melittins

Reversed phase HPLC separates molecules on the basis of overall hydrophobicity. The labeling of an amino group with the spin label makes the protein more hydrophobic. The resulting derivative thus interacts slightly stronger with the stationary phase and elutes later than the unmodified protein.

In order to obtain baseline separation of all 16 possible reaction products, a 25 cm × 4.6 mm RAININ Dynamax C18 column (Rainin Instrument Company, Inc., Woburn, MA) with 5-micron particles and 100-Å pore size was used. The built-in precolumn (1.5 cm) had the same packing material. Solvent A was water containing 200 mM $NaClO_4$ and 0.1% phosphoric acid, pH 2.1, and solvent B was acetonitrile with 200 mM $NaClO_4$. A VARIAN 5060 liquid chromatograph was programmed to perform a linear gradient of 50% to 100% B in 1 hour with a flow rate of 1 ml/min. The chromatogram was monitored with a Hewlett Packard 1040M diode array detector (Hewlett Packard Analytical Group, Palo Alto, CA).

The relatively high sodium perchlorate concentration is important because, under the same conditions but with only 100 mM perchlorate, significantly broader peaks were observed. Other solvent modifiers recommended for protein separation, like trifluoroacetic acid, were also tested without success. The action of sodium perchlorate is twofold: it suppresses ionic interactions between positively charged melittin sidechains and residual silanol groups of the column, and it dramatically increases the solubility of melittin in the organic phase. A three-dimensional representation of the elution pattern between 41 and 59 minutes and over a wavelength range of 240 to 350 nm is shown in Figure 1. The number above each

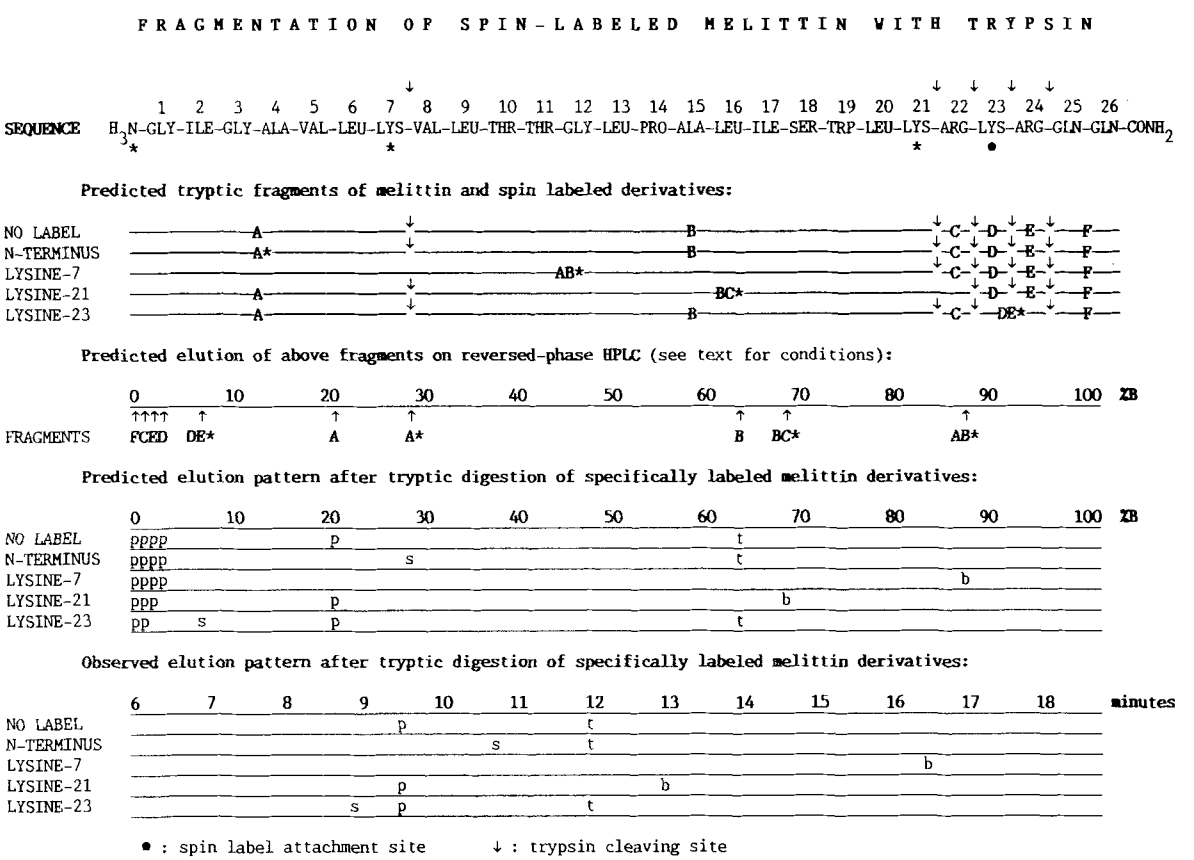



Fig. 2. Summary of the trypsin digestion experiments to determine the primary structure of the spin-labeled melittin derivatives. The amino acid sequence of melittin is shown at the top. An asterisk (*) indicates a potential attachment site for a spin-label, and the arrows (↓) show the trypsin cleavage sites. Whenever a lysine is labeled, this site is no longer cleaved. The theoretical fragments for melittin and of all melittin derivatives with only one spin label attached are shown followed by a summary of the predicted and observed HPLC elution patterns for all melittin derivatives.

peak indicates the residue number of the lysine amino group to which the spin label is attached. N indicates that the label is attached to the N-terminus. The methods used for these assignments are described below.

Figure 1 shows that all possible reaction products are resolved. The integrated intensities of the peaks at 280 nm are consistent with the statistically predicted relative amounts of each reaction product assuming equal reactivity of all sites. The amount of melittin with all four sites labeled is very low under these labeling conditions and the corresponding peak not visible. However by reacting an excess amount of spin label with melittin, only one peak at the indicated position at the very end of the chromatogram was obtained. This melittin with all four amino groups reacted is very hydrophobic and not water-soluble.

After neutralizing the collected fractions with NaOH most of the acetonitrile was removed with a stream of argon. Subsequently they were loaded on a PD-10 desalting column (Pharmacia, Piscataway, NJ) eluted with 1 M acetic acid to remove the perchlorate and phosphoric acid, and then lyophilized. The resulting white powder was resuspended in distilled water at a concentration of $\simeq$ 3 mg/ml and stored at $-20°C$. These stock solutions, when reinjected, gave single peaks with $>$ 95% purity.

## Assignments of Fractions

### *Determination of the number of spin labels attached to melittin derivatives*

At 280 nm, only the tryptophan side chain of melittin has significant absorbance, but at 250 nm the UV absorbance of the spin label dominates. From the ratio of $OD_{250}/OD_{280}$ or by comparing the spectral shape of the eluting peaks with simulated spectra of the sum of the tryptophan spectrum and 1, 2, 3, or 4 spin label spectra, the ratio of spin label to melittin is unambiguously determined.

### *Determination of the label sites for melittins with one label attached*

To determine which labeled melittin derivative corresponds to a given HPLC fraction, a trypsin digestion of the unlabeled melittin and of all fractions with one label per melittin was performed. The protease trypsin cleaves after positively charged amino acids

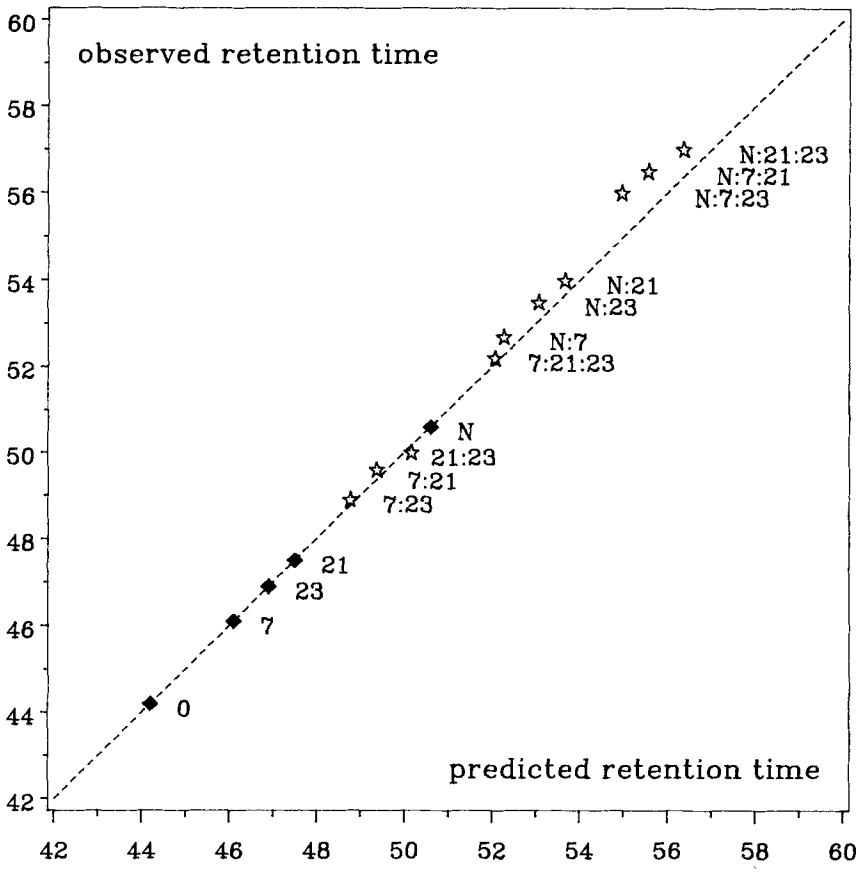



Fig. 3. Correlation between observed HPLC retention times and their predicted values according to the model described in the text. ♦, Positions of unlabeled and monolabeled melittins used to predict the positions of the multilabeled fractions (☆).

(lysine, arginine), and because a labeled lysine is no longer a cleaving site the digestion of each spin-labeled melittin leads to a unique fragment pattern as analyzed by HPLC. It is possible to predict the relative elution order of the fragments from their amino acid composition,[33] and as an additional control, these predictions were compared with the observed elution pattern. Four types of fragments are distinguished according to their UV spectrum: type p, peptides with absorbance only in the low UV region which contain no spin label and no tryptophan; type t, peptides containing a tryptophan and no spin label; type s, peptides containing only a spin label and no tryptophan; type b, peptides containing both spin label and tryptophan. The sequence of melittin and the expected fragments for unlabeled and monolabeled melittins are summarized in Figure 2.

Unlabeled melittin is expected to be cleaved into six fragments A–F but there are only two major fragments: A(1–7) and B(8–21). They are predicted to elute at approximately 22% B and 64% B, respectively. A is type p and B is type t. The small C terminal fragments (C–F) are very hydrophilic and are expected to elute very early in the chromatogram. It will be shown that these fragments are not needed for an unequivocal assignment of the label site. For the N-terminal labeled melittin the same pattern is predicted except that fragment A carries now a spin label (A*) and should elute slightly later than fragment A. A* is of type s. Melittin labeled at lysine-7 gives only one large major fragment AB*(1–21) which carries a spin label and a tryptophan (type b) and is predicted to elute very late at ≃ 89% B. For lysine-21-labeled melittin, the unmodified peptide A and a new fragment BC*(8–22) should result from the digestion. This fragment is predicted to elute slightly after fragment B at ≃ 70% B and is of type b. If the spin label is attached to lysine-23, the unmodified fragments A and B are obtained and in addition the dipeptide DE*(23–24) with a spin label attached. This type s peptide should elute very early at 8% B. Thus, each derivative is expected to produce a unique set of peptides readily distinguishable by UV absorption and elution pattern.

Trypsin digestions were performed by mixing 30 μg melittin with 10 μg trypsin in 100 μl Tris buffer, pH 8, 10 mM $CaCl_2$. After 2 hours at room temperature the digestion was complete, and the fragments were separated with HPLC. The same solvents as for purification of the spin-labeled melittins were used but

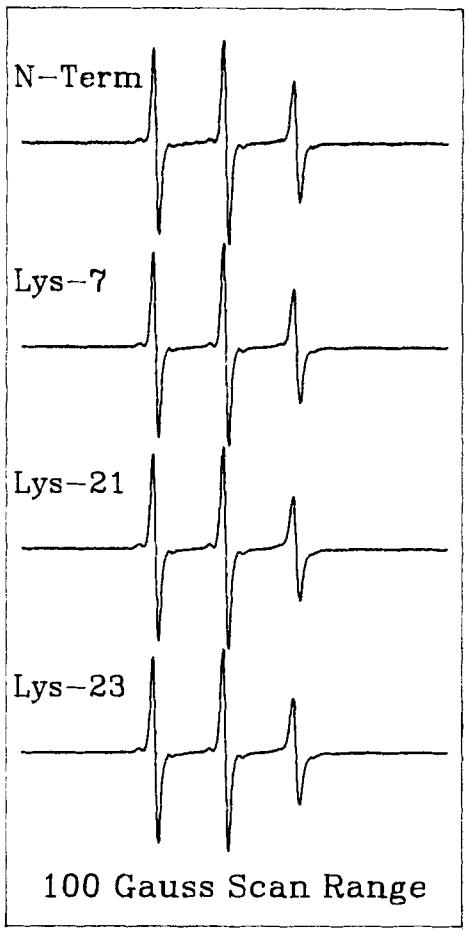



Fig. 4. Comparison of the EPR spectra of spin-labeled melittin in the monomer conformation. The spin label was attached either to the N-terminus (N) or to one of the three lysines (7, 21, 23) as indicated. Each sample contained 0.75 mg/ml spin-labeled melittin in distilled water. Four 30-sec scans with a time constant of 0.008 sec were signal averaged for each spectrum.

here the gradient was 0% to 100% B in 15 min followed by 6 min at 100% B. For each of the monolabeled melittin derivatives the peptide pattern following proteolysis unambiguously identified the derivative by comparison with the predicted results discussed above as shown in Figure 2. The only deviation from the predicted results was that all peptides eluted at a higher percentage B, probably due to the rather steep gradient of 6.6% B/min. It must be emphasized that the primary basis of the positional assignment of the spin label is the type of peptides produced by proteolysis and not the absolute position of the peptides in the chromatogram. Nevertheless, the predicted and observed chromatograms are remarkably close and serve to confirm the assignments.

### *Determination of the label sites for melittins with more than one label attached*

The effect of an attached spin label on the retention time of melittin does depend on the label site. Without these secondary interactions all fractions with the same number of spin labels per melittin would coelute. Having determined the position of the label in each of the monolabeled derivatives, we can determine from the chromatogram the effect of a label at each site on the retention time relative to unlabeled melittin. From HPLC theory it can be assumed that these shifts are additive for melittins with more than one label attached.[33] With this simple concept, all multilabeled peaks can be assigned by simply adding the shifts in retention time of the corresponding monolabeled melittins. The identification of the peaks of multilabeled melittin derivatives as shown in Figure 1 were obtained in this fashion. Figure 3 shows the excellent correlation between observed and predicted retention times.

### *Determination of the hemolytic activity of melittin and its spin-labeled derivatives*

An aliquot of the stock solutions of purified melittin and its derivatives were diluted 10× with hemolysis buffer (20 mM Tris, 150 mM NaCl, pH 7.4). Ten microliters of each of these solutions were analyzed with HPLC, and the exact concentration of protein was then determined by integration of the chromatogram at 280 nm corrected for the residual absorption of the spin label at this wavelength. Subsequently another dilution was made to achieve equal protein concentration in all samples. Hemolysis was then carried out at 37°C following the procedure of Hider et al.[17]

## RESULTS

### Hemolytic Activity

All purified spin-labeled melittin derivatives were fully active in the lysis of red blood cells when compared to purified unlabeled melittin using the hemolysis assay. Unpurified Sigma melittin, however, showed a 3 to 4 times higher activity if the protein concentration was normalized to the same area of the melittin peak. This indicates that significant hemolytic activity also originates from the impurities such as N-formyl-melittin and phospholipase $A_2$.

### Melittin in Aqueous Solution

The EPR spectra at low ionic strength of all four mono spin-labeled melittin derivatives are shown in Figure 4. They all show three sharp lines indicating very rapid and independent motion of the individual segments. This agrees well with the established model of an unfolded, predominantly random coil structure. From the lineshape, we estimate the rotational correlation time of the nitroxide group to be 1–2 nsec. Very small differences, however, do exist between the different label sites as can be seen in the relative height of the high field line. The relative mobility of the labeled segments is: N-terminus > lysine-7 ⩾ lysine-23 ⩾ lysine-21.

Upon salt-induced transition to the tetramer all labels become more immobilized. To analyze the line-

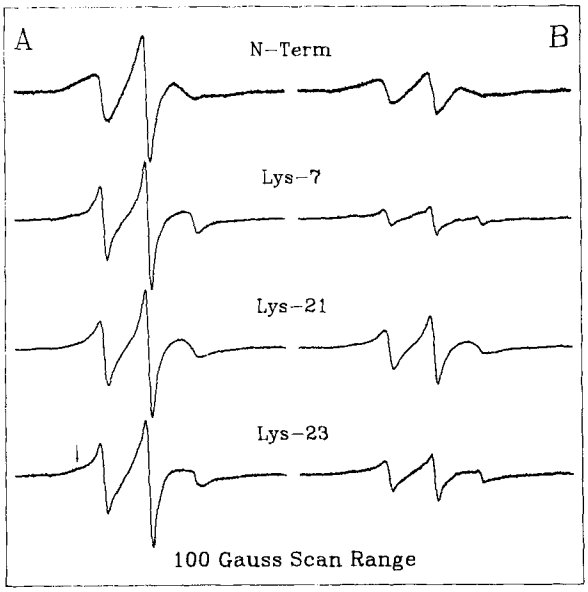



Fig. 5. Comparison of the EPR spectra of tetrameric melittin in high salt concentration. Each sample contained 1.5 mg/ml spin-labeled melittin, 2 M NaCl, and 100 mM phosphate, pH 7. The spectra to the left in addition contained 20 mg/ml unlabeled melittin (**A**); here each tetramer contains less than one spin. The broader lines and reduced amplitude in the spectra to the right (**B**) is a result of the magnetic interaction of the four individual spins per tetramer. Four 30-sec scans with a time constant of 0.008 sec were signal-averaged.

shapes of the tetramer spectra we have to consider two separate cases. If the tetramer is formed only with labeled melittin, the local "spin concentration" in the tetramer may reach 500 mM, and considerable additional broadening of the EPR spectra has to be expected. With such a high spin concentration, the individual spins may interact by Heisenberg exchange due to diffusion-controlled collisions between nitroxide groups and magnetically by dipolar interaction through space. However, if we add an excess amount of unlabeled melittin to the solution, each spin-labeled melittin will normally form a tetramer with three unlabeled melittins, and spin interaction can be excluded. In this case the spectrum indicates the true mobility of the label. Fig. 5 shows the EPR spectra of the different tetramers in the presence (Fig. 5A) and absence (Fig. 5B) of an excess of unlabeled melittin at room temperature. First we will focus on the cases without spin interaction (Fig. 5A). From the spectra with excess unlabeled melittin we estimate the rotational correlation times to be between $\simeq$ 14 nsec for the labeled lysines and $\simeq$ 20 nsec for the label attached to the N-terminus. To determine to what extent local mobility of the spin label and global mobility of the whole tetramer contribute to the spectrum, we repeated the measurements in a solution of higher viscosity by adding 30% sucrose to the solution. Because $d\tau/d\eta$ is proportional to $a$,[3] increases in viscosity reduce the overall motion of the tetramer dramatically, whereas the local motion of the label relative to the protein is less affected. Figure 6 shows the spectra in 30% sucrose ($\eta \simeq 3$ cP at 22°C). It is immediately evident that the motion of the N-terminus is now considerably immobilized and anisotropic, but the labeled lysine side chains remain more mobile. This behavior can be explained by the fact that for spin-labeled lysines the label is attached to the protein by a flexible aliphatic chain. At the N-terminus this chain is much shorter and the attachment of the label with a normal peptide bond permits the formation of an additional hydrogen bond between the C=O of the label and N-H of alanine-4 thus extending the rigid $\alpha$-helix by an additional residue. An analogous situation, but with a different spin label attached to the C-terminus of poly-$\gamma$-benzyl-$\alpha$,L-glutamate, was described by Wee and Miller.[49] They obtained very similar spectra that could be described with a anisotropic motion.[22] Figure 7 shows the structure of the spin-labeled N-terminus according to this model. In this conformation, the nitroxide radical is rigidly attached to the $\alpha$-helix backbone. Even the rotation of the nitroxide ring is slightly hindered, as judged by a space-filling model of the labeled melittin. The correlation time of this label is therefore a very good estimate for the correlation time of the entire tetramer.

The effect of spin interaction in the labeled tetramers is very dramatic as can be seen in the spectra in Figure 5B. As a first measure of the effect, we compare the EPR signal amplitude of the same number of spins in the presence and absence of unlabeled melittin. The largest effect can be seen with the spin label attached to lysine-7. Here the spin interactions cause a 78% reduction in height of the centerline. For the other labels the observed decrease in line height due to spin interaction broadening are 67% for N-terminus, 46% for lysine-21, and 62% for lysine-23.

As mentioned above, the spin interaction leading to the increased linewidth and decreased intensity could be either Heisenberg exchange or dipolar interaction or both. To get an idea of the relative importance of their contributions. we examined the spectra of the tetramers rapidly frozen in solution at −105°C where all Heisenberg exchange contributions are eliminated. With excess unlabeled melittin present, completely immobilized typical powder spectra are obtained. An example is given in Figure 8A. With only labeled melittin, however, the lineshapes are completely different and indicate large dipolar interactions (Fig. 8B). For the case of only two interacting spins it is possible to estimate the distance between the two radical centers from the lineshape of the immobilized spectrum,[25] but because in this case up to four spins may interact and the individual interspin distances may vary due to the flexibility of the lysine chains and relative arrangements of the subunits, the analysis is not so straightforward. Qualitatively the lysine-7 spectrum looks very similar to the spectrum of a tetraradical with interspin dis-

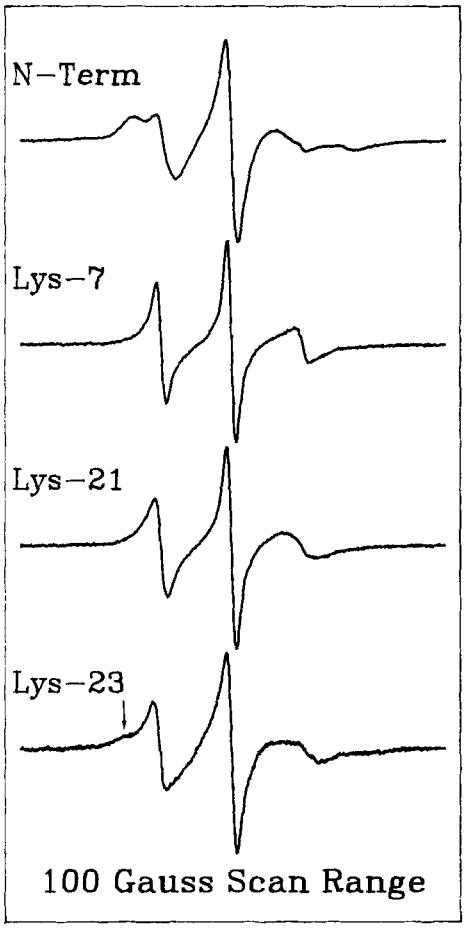



Fig. 6. Comparison of the EPR spectrum of tetrameric melittin in high viscosity. Each sample contained 0.75 mg/ml spin-labeled melittin, 25 mg/ml unlabeled melittin, 40 mM phosphate, pH 7, and 30% sucrose. Measuring conditions as in Figure 3.

tances between 13 and 23 Å.[27] In agreement with the results at room temperature, lysine-7 shows the strongest interaction.

### Melittin Bound to Membranes

Melittin binds strongly to both neutral and to negatively charged phospholipid membranes. Figure 9 shows the EPR spectra of spin-labeled melittin bound to neutral (Fig. 9A) or negatively charged (Fig. 9B) bilayers. The absence of narrow lines in all spectra clearly indicate 100% binding in each case and much reduced motion at each position. To probe for possible aggregation of melittin to dimers or tetramers in membranes, efforts were made to detect spin interaction analogous to the observations in the aqueous tetramer. For all labeled melittins there was no difference in signal shape and amplitude between samples with and without excess unlabeled melittin when bound to neutral or negatively charged membranes. To further test if high salt concentration has an effect on the aggregation state of melittin in membranes as it does in solution, the experiment was repeated in 2 M NaCl, 100 mM phosphate, pH 7, in the aqueous phase. Also, no indication of spin interaction was evident (data not shown). From these findings we conclude that in all these systems a very high fraction of membrane-bound melittin is monomeric. If some of the melittin is aggregated it must be less than 5%, which is the estimated reproducibility in resonance amplitude measurement.

The mobility of the spin label due to membrane binding is always less in negatively charged membranes than in the neutral membranes, presumably because of increased electrostatic interaction between the negatively charged lipids and the positively charged groups of melittin. In all cases, the label at the N-terminus was considerably more immobilized than the labels at any one of the lysines. This indicates that the postulated hydrogen bond between the label and alanine-4 is probably preserved upon membrane binding.

To determine if the individual labels are buried in the membrane or face the aqueous phase, we added 50 mM chromium oxalate to the aqueous phase. This hydrophilic paramagnetic compound will shorten the electron relaxation time and thus broaden the EPR spectrum of the nitroxide if it undergoes direct colli-

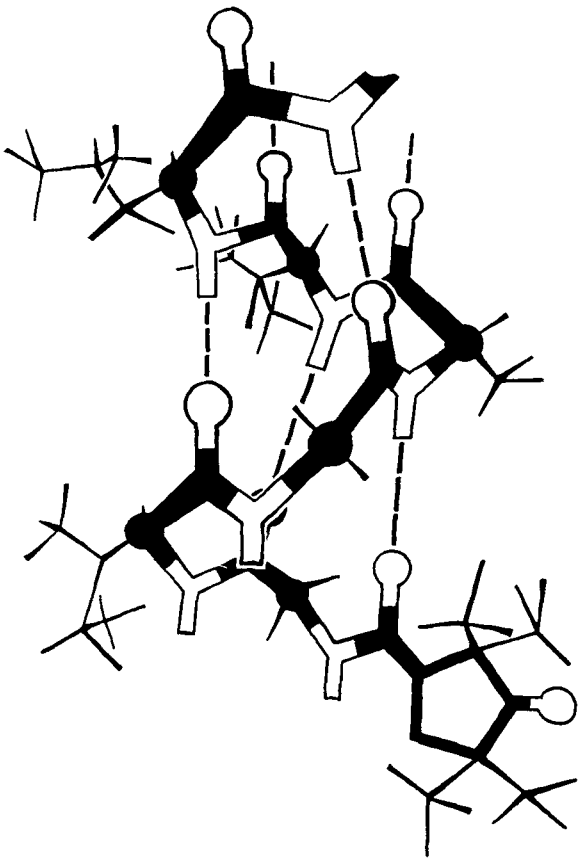


Fig. 7. Postulated three-dimensional structure of the spin-labeled N-terminus of melittin. Note the additional α-helical-type hydrogen bonding between the spin label and alanine-4. This hydrogen bond causes the very rigid attachment of the nitroxide in the tetramer.

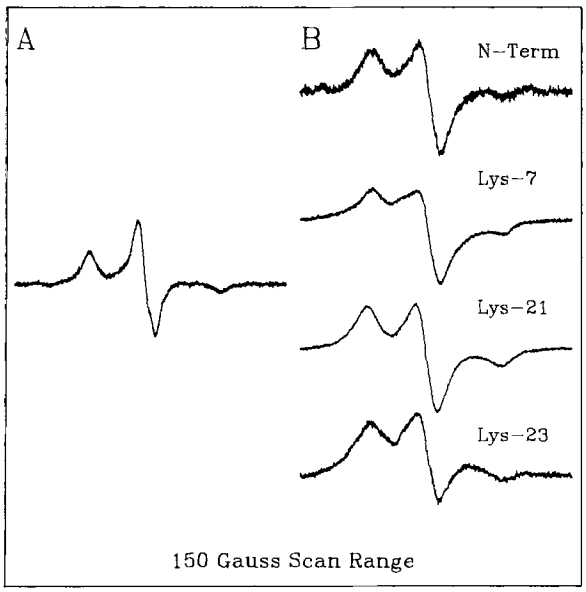



Fig. 8. EPR spectra of tetrameric melittin as in Figure 7 but now in a rapidly frozen solution at − 105°C. The spectrum to the left (**A**) contains excess unlabeled melittin and indicates completely immobilized, randomly oriented nitroxides. All spectra to the right (**B**) are broadened due to dipolar interaction in the tetralabeled tetramer. Note that the scan range is 150 gauss.

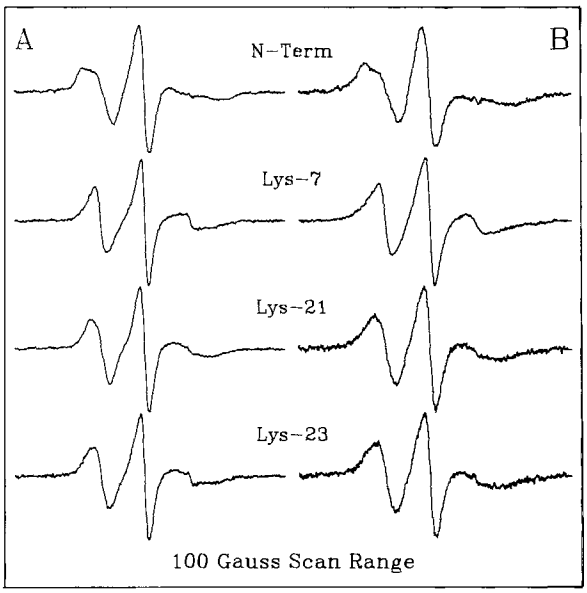



Fig. 9. EPR spectra of spin-labeled melittin bound to neutral (**A**) and negatively charged (**B**) phospholipid bilayer membranes. Each sample contained 0.75 mg/ml spin labeled melittin, 40 mM phosphate buffer, pH 7, and 40 mg/ml phospholipid. In A, the phospholipid was DOPC, but B was obtained with a mixture of DOPC:POPA = 9:1. We averaged 32 scans (time constant = 0.032 sec).

sion with the nitroxide. Because chromium oxalate is charged, it is not expected to enter the bilayer and will undergo collisions only with spin labels exposed to the aqueous phase. Figure 10 shows the spectra of melittin bound to neutral phospholipid bilayer membranes with and without chromium oxalate. All spin labels are at least partially accessible to the broadening agent, consistent with a surface location for the bound melittin as opposed to one in which the polypeptide is buried within the bilayer. From the change in signal amplitude we see that lysine-7 shows the largest change (61% reduction). It is therefore most exposed to the aqueous phase. Lysine-23 (54% reduction), N-terminus (52% reduction), and lysine-21 (37% reduction) are slightly more buried. As a control we added chromium oxalate to monomeric melittin in the absence of membranes. Under these conditions. the signal is reduced to less than 1%. Chromium oxalate has no effect on an androstane spin label (N-oxyl-4',4'-dimethyloxazolidine derivative of 5α-androstan-3-one-17β-ol)[20] where the nitroxide group is buried in the hydrophobic interior of the membrane. A more quantitative study employing saturation recovery EPR spectroscopy confirmed that lysine-7 is most exposed and indicates that lysine-23 is most buried in the membrane and that the N-terminus exists in two conformations with different accessibility.[1]

## DISCUSSION

The method we present here of attaching a spin label to different locations on the bee venom protein, melittin, provides a way of obtaining highly pure, specifically labeled melittin in quantities large enough for EPR measurements using a loop-gap resonator. The perturbation of one label attached to melittin clearly does not change the hemolytic activity and properties of melittin. This is in agreement with other studies. DeGrado and co-workers showed that a synthetic structural analogue of melittin with little sequence homology and no charge at position 7 is hemolytic.[8] Even melittin with four of the six positive charges removed by acylation of all amino groups shows comparable hemolytic activity[14] and shows voltage-dependent pore formation and resolved pore state conductance.[15] In addition, melittins with two attached labels might also be useful. They should show spin interaction (exchange, dipolar) that can be used for distance measurements between the two labels in the random coil monomer, in the tetramer when mixed with excess unlabeled melittin in high salt, in membrane-bound melittin, or in the α-helical monomer in nonaqueous solutions. One has to be aware, however, that with each spin label attached, small changes may be introduced in the molecular organization of melittin so that the results with multilabeled melittins may not accurately reflect the picture of the original protein. However, preliminary measurements show that the double and triple spin-labeled melittins are at least as hemolytic as native melittin.

### Melittin in Solution

At low ionic strength we obtained rotational correlation times that indicate a high degree of segmental

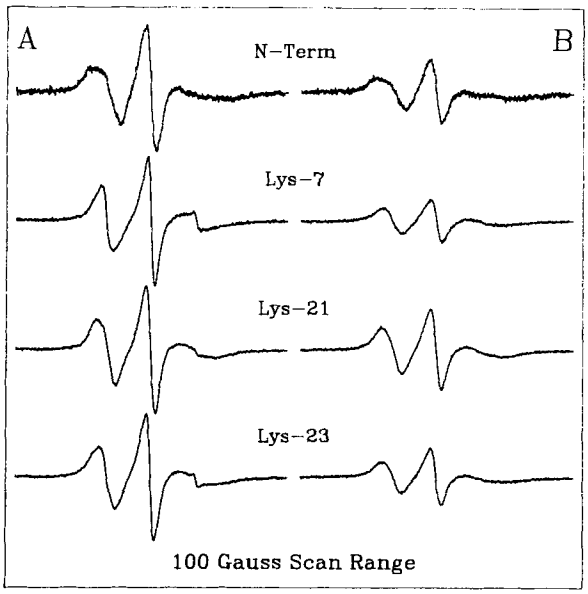



Fig. 10. EPR spectrum of spin-labeled melittin bound to neutral phospholipid bilayer membranes in the absence (**A**) and presence (**B**) of 50 mM chromium oxalate (same conditions as in Fig. 7). Chromium oxalate does not affect a spin-label buried deep in the membrane but broadens the spectrum of a spin-label exposed to the aqueous phase. The intermediate results observed indicate positioning of the spin labels in the headgroup region of the membrane.

flexibility. The motions are slightly faster towards the tails and slower within the protein chain. This is consistent with an unfolded random coil structure where the "loose ends" have higher mobility than the center part. This situation is reversed in the tetramer. Here the spin labels attached to a lysine chain show more independent local motion, whereas the N-terminus is more immobilized due to a presumed intrachain hydrogen bond. The spectra of the labels in the tetramer indicate at least two spin populations of different mobility (Figs. 5, 6). This is particularly evident with the label at lysine-23. The less mobile of these (↓, in Figs. 5, 6) may be due to hydrogen bonding between the label amide linkage and a nearby arginine (position 22 or 24) or glutamine (position 26) or even due to hydrogen bonding to another subunit in the tetramer. It is, however, not yet clear as to whether or not the highly charged C-terminal end is α-helical in the solution tetramer. In the crystal it is α-helical.[40] More recent Raman measurements by Vogel and co-workers indicate 76% α-helix in the melittin tetramer and, disregarding the proline bend, the non α-helical part is probably the highly charged C-terminus.[47] The addition of 100 mM phosphate boosts this value to 92%, which corresponds to 24 residues and is identical to the crystal structure. It is not possible to determine the structure of the C-terminus from our data, but from comparison with the monomer spectra it can be concluded that every part of melittin is definitely more rigid and structured in the tetramer.

We can compare our results with rotational correlation times from fluorescence measurements of tryptophan-19 or from an anthraniloyl label attached to lysine-21. The most recent measurements by Lakowicz et al. show correlation times of 1.7 nsec for monomeric melittin and 3.4 nsec for the tetramer as obtained by multifrequency phase modulation fluorometry of tryptophan-19.[28] Their number for the tetramer does not agree with our results. In an earlier, more detailed analysis from the same laboratory, N-methyl-anthraniloyl-melittin was analyzed with three independent correlation times.[30] A very short correlation time that was always in the range of 0.14 nsec to 0.27 nsec was attributed to local mobility of the label. An intermediate correlation time indicated "domain" mobility and a longer one was believed to reflect the overall motion of the protein or tetramer aggregate. In the monomer they measured 0.93 nsec (amplitude 33%) for the intermediate motion but detected also a longer one of 3.6 nsec with very low (7%) amplitude. Their numbers for the tetramer are 3.45 nsec (53%) and 13.9 nsec (19%) and in melittin bound to DMPC (1:100) they obtained 3 nsec (36%) and 47.4 nsec (38%). The anthraniloyl label in these measurements was attached to lysine-21, and the results should therefore be directly comparable to our measurements with melittin labeled at this position. Our measurement of $\simeq$ 1 nsec for the monomer agree very well with the intermediate motion of 0.93 nsec. At lysine-21 in the tetramer we observe only the slowest component ($\simeq$ 14 nsec). This is, however, still faster than the overall rotation of the melittin tetramer. The label at the N-terminus is more rigidly attached and shows a correlation time of $\simeq$ 20 nsec. Theoretical calculations of the rotational correlation time for the tetramer need, in addition to the size of the tetramer, some assumption of the number of bound phosphate ions and the size of the hydration shell, which might be quite large for this highly charged protein (12–16 positive charges). An ideal spherical protein with a correlation time of 20 nsec in our solvent system would have an equivalent radius of 25 Å. This is not unreasonably high. The length of the α-helical monomer in organic solvent, which is expected to have a conformation very similar to one subunit of the tetramer is 54 Å and has a radius of 13 Å as measured by dielectric dispersion measurements.[37] In summary, our measurements of the spin label attached to lysine-21 are in overall agreement with measurements obtained with an anthraniloyl label attached to this site. We conclude, however, that even the longest rotational correlation time measured at this location does not reflect the overall motion of the tetramer. Only the label attachment to the N-terminus is rigid enough to provide a reliable estimate of the rotational correlation time of the tetramer.

Whenever a tetramer is formed with four spin-labeled melittin subunits, strong spin interactions are observed. Spin interactions are either dipolar (through

space) or by direct collision (Heisenberg exchange). Dipolar interaction depends on the reciprocal of the cube of the spin-label separation and is completely averaged out by fast isotropic motion.[31] Incomplete motional averaging of the dipolar interaction due to the slower rotation of the tetramer causes linebroadening.[46] If the solution tetramer is the same as the crystal structure, it is unlikely that direct nitroxide collisions could occur because the amino groups are relatively far apart. This is particularly true for the spin labels that are rigidly attached to the N-terminal ends. Thus, one would assume that the primary interaction is dipolar, and we have shown that the nitroxides are in fact close enough to give rise to strong dipolar coupling in the solid state. On the other hand, the solution structure may not be as rigid as the crystal structure, and sufficient relative motion of the helices could give rise to Heisenberg exchange interaction. We cannot exclude this possibility on the basis of the present data.

### Melittin Bound to Membranes

The structure of membrane-bound melittin is mainly $\alpha$-helical and probably very similar to one subunit in the aqueous tetramer and contains approximately 76% $\alpha$-helix.[47] But here the basic agreement in the literature ends. Experiments designed to address the question of aggregation state and orientation lead to contradictory conclusions. From fluorescence energy transfer from tryptophan-19 to a melittin with a modified tryptophan, Vogel and coworkers proposed a detailed model of tetrameric aggregation,[47] whereas Hermetter and Lakowicz concluded from fluorescence energy transfer between tryptophan-19 and a N-methyl-anthraniloyl residue attached to lysine-21 that it is definitely monomeric.[16] Fluorescence studies with melittin dansylated at the N-terminus confirm this result,[11] but studies by Talbot and co-workers with a N-bromosuccinimide oxidized tryptophan-19 melittin indicate aggregation of melittin bound to membranes in the presence of NaCl.[38] Others support the monomeric model for more indirect reasons.[29, 35, 39, 42] Our measurements of spin interaction in melittin bound to phospholipid bilayers strongly supports the monomer model. The tetramer model proposed by Vogel and coworkers is particularly inconsistent with our results.[47] In the aqueous tetramer, all amino groups are more or less on the outside surface and all of the spin-labeled derivatives show strong spin-spin interaction. In the model of the membrane-bound tetramer proposed by Vogel and co-workers, the hydrophobic faces of the helices are pointing outwards and the hydrophilic side chains to the channel interior.[47] In this "inverted" tetramer the spin labels should be even closer together than in the aqueous tetramer. Especially for lysine-7, very large spin interaction would be expected. Spin-spin interaction has not been observed in any labeled melittins in the membrane-bound state even in the presence of high salt; thus, we conclude that membrane bound melittin is monomeric. Since electrostatic interactions between monomers is entirely repulsive any oligomeric structure must be stabilized by hydrophobic effects and/or hydrogen bonding. Given this constraint, it is difficult to imagine a stable structure in which the nitroxides can be further apart than in the solution tetramer.

Bilayer measurements show that the conductance of the melittin channel increases with the fourth power of melittin concentration,[45] suggesting that the active channel may be tetrameric. This is not in contradiction with our results because only a very small fraction of all membrane-bound melittin may form a tetramer, and we also do not exclude the possibility of melittin aggregation in the presence of a transmembrane potential.

There is also little agreement about the orientation of the $\alpha$-helical segments of melittin relative to the membrane surface. Podo,[34] Vogel,[48] Coddington,[6] Dufourc,[9] and their co-workers postulate that the $\alpha$-helix axis is more or less perpendicular to the membrane surface. In these models, melittin penetrates deeply with either the N-terminal part or the proline bend, at least half-way through the bilayer, and lysine-7 is buried in the membrane. Others conclude that melittin binds to membranes with its axis parallel to the membrane surface and lysine-7 is most exposed to the aqueous phase.[5,7,42] A more differentiated model shows a binding parallel to the surface, which in turn changes to a perpendicular orientation when a transmembrane voltage is applied.[4,24] The fact that all label sites are at least partially accessible to the aqueous phase as judged by collision with chromium oxalate leads us to believe that melittin is bound to the surface of the membrane. Our method tends to underestimate the water accessibility. Because a native lysine side chain is more hydrophilic than a spin-labeled one, we can expect them to be even more exposed to the water than estimated from our EPR measurements. From the differences in accessibility to chromium oxalate we conclude that lysine-7 is pointing towards the aqueous phase, and lysine-21, lysine-23, and the N-terminus are approximately in the neighborhood of the headgroups. Brown and coworkers used paramagnetic linebroadening of NMR lines of melittin bound to spin-labeled deuterated dodecylphosphocholine micelles and obtained similar results.[5] They locate lysine-7 at the surface of or exterior to, the micelle. The N-terminus, lysine-21, and lysine-23 are found at, or slightly exterior to the phosphate group of the detergent molecules. This is in excellent agreement with our results. These relative accessibilities strongly support a model in which the hydrophobic faces of the helical segments face the membrane, as postulated by Terwilliger.[40]

These conclusions of course do not rule out the possibility of a voltage-induced transmembrane orientation of melittin. This model, in which an $\alpha$-helical dipole and/or charged residues interact with an external electric field, causing conformational and orien-

tational changes of the protein, is of great biological significance. Such mechanisms are indeed thought responsible for voltage-gated channels.[18] We are currently addressing voltage-dependent changes in membrane-bound spin-labeled melittin and investigating the molecular mechanism of voltage-dependent conformational changes.

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