*Biochimica et Biophysica Acta*, 1069 (1991) 77–86

*ADONIS* 000527369100366L



BBAMEM 75382

# A combined study of aggregation, membrane affinity and pore activity of natural and modified melittin

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(Received 3 April 1991)

Key words: Lipid–peptide interaction; Bilayer permeabilization; Melittin, formylated; Melittin, acetylated; Melittin, succinylated; Tryptophan modification

**The pore activity of melittin and several chemically modified derivatives has been investigated using conductance measurements on planar lipid bilayers and marker release from small unilamellar vesicles. The modifications included N-terminal formylation, acetylation, succinylation and modification of the tryptophan residue. All of the compounds showed bilayer permeabilizing properties, though quantitative differences were evident. These comprised changes in the voltage dependence of the conductance, in the single-pore kinetics, in the concentration of aqueous peptide required to induce a given pore activity and in the apparent 'molecularity' reflected by the power law of its concentration dependence. A strong tendency for disrupting bilayers was not always correlated with strong pore activity. For a better understanding of these results, measurements of pore activity were complemented by studying the aggregation behavior in solution and the water-membrane partition equilibrium. Modifications of charged residues gave rise to significant changes in the aggregation properties, but had virtually no influence on the partition coefficient. The latter decreased strongly, however, as a result of tryptophan modification. Analysis of the isotherms was consistent with the assumption that the arginine residues in melittin do not contribute very much to charge accumulation at the immediate membrane/water interface.**

## Introduction

Honeybee melittin is a small natural peptide (26 amino acids) that has been widely studied as a model for various aspects of protein–lipid interactions (see Ref. 1 for a recent review). It has a pronounced amphiphilic nature and belongs to a group of peptides that induce voltage-dependent conductance in membranes [2] apparently through aggregation of several monomers to form pores. However, very few melittin molecules do ordinarily exist in an open pore state. This is much too little for most experimental techniques to get a direct insight into the actual channel structure and function. On the other hand, titration of an aqueous peptide solution with lipid vesicles results in a substantial circular dichroism signal indicating that melittin strongly partitions into the lipid bilayer environment where it assumes a helical structure [3]. How a small part of this apparently non-pore form of the bilayer associated peptide will be turned into a voltage-sensitive porous aggregate is the crucial question regarding the molecular mechanism of the pore activity. In aqueous solution at high salt concentrations the existence of a tetrameric aggregate is well established which is composed of amphipathic monomeric helices with their hydrophobic faces pointing to the inside and the opposite hydrophilic faces presenting themselves to the water phase [4]. Vice versa, one may model a melittin pore in the lipid bilayer moiety as a kind of barrel stave collection of membrane spanning helices (not necessarily only four) with the polar faces lining a water-filled pore and the nonpolar faces contacting the surrounding lipid.

Abbreviations: DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; NBS, *N*-bromosuccinimide; HNBB, 2-hydroxy-5-nitrobenzylbromide; HPLC, high-performance liquid chromatography; CD, circular dichroism; BLM, black lipid membrane.

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Evidence has accumulated that upon association with lipid bilayers the melittin helices show no perceptible tendency to aggregate and do not span the membrane, but lie on the surface with the polar side directed towards the water [5,6]. Such studies were performed without any transmembrane potential, and so it appeared reasonable to assume that the role of the potential was to bring the peptide into the transmembrane orientation required to form a pore [7]. This would implicate the N-terminal Gly and the Lys 7 as "gating charges". Nevertheless, it was shown later that similar voltage-dependent conductance was also obtained with an acetylated derivative in which these charges were blocked [8]. Investigators were thus led to consider the dipole moment of the helix as providing the gating effect. Subsequent studies with synthetic peptides selectively blocked at the two charges on the N-terminal end of the molecule showed that these charges are not required for gating per se but that they do modulate the conductance behavior [9]. In addition, recent studies in our laboratory suggest that the switch from a membrane-surface state to a transmembrane state precedes the gating event [10]. The voltage sensitivity of the various steps, including partitioning, attainment of a membrane-spanning conformation, aggregate formation and opening of the pore, is not yet fully known.

Similar uncertainties remain concerning the aggregation behavior of the peptide. Energy transfer studies of various chemical modifications of melittin have given conflicting results. In the majority of the experiments, aggregation was either not detected or arose only at high melittin concentrations [11–13]. However, a recent calorimetric study was interpreted as indicating that appreciable aggregation does occur at high ionic strength [14]. Indeed, it is likely that high ionic strength is necessary to screen the cluster of four positive charges near the C-terminus of the peptide, and this will be true for both membrane-associated and aqueous melittin. But the effect of the tryptophan modification is still unclear, since even small modifications give relatively large changes in behavior.

In view of the diverging results obtained so far with this molecule, it appears necessary to have a somewhat more systematic study of the effects of the various modifications to which one can subject the melittin molecule. In this study we investigate the roles of the charged groups and the tryptophan in melittin by examining unmodified melittin and some of its derivatives. Our goal is to characterize these molecules with respect to the thermodynamics of the processes that must be considered in any analysis of the action of melittin: aggregation in aqueous solution, partitioning into the membrane environment and pore formation in model membranes. By studying several modifications we are better able to correct for artefacts arising from the modifications themselves. We utilize lipid vesicles and planar black lipid membranes (BLM) and make use of circular dichroism (CD), analytical ultracentrifugation, vesicle permeation and conductance techniques in characterizing the compounds.

## Materials and Methods

### *Materials*

Lipids (POPC, DOPC and DMPC) were from Avanti (Pelham, AL, U.S.A.). Melittin was from Sigma (St. Louis, MO, U.S.A.) or from Serva Heidelberg, F.R.G.), both samples yielding comparable results. It was passed through a Diaflo YM10 membrane to remove contaminating phospholipase. Material used for vesicle permeabilization or BLM studies (apart from acetylated and Trp-modified melittin) was further purified by HPLC (see Ref. 15 for details).

Formylated melittin was obtained as a minor component in the HPLC run of the original melittin sample. It ran slightly more slowly than the main component during electrophoresis on cellogel strips (Serva) in formic acid-urea (see Ref. 16 for details).

Acetylation and succinylation were done according to standard procedures, with a 45-fold molar excess of acetic anhydride over reactive amino groups and a 15-fold excess of succinic anhydride, respectively. Acetylation was performed in phosphate buffer (pH 7) and succinylation in Tris buffer (pH 8) with NaOH added to maintain the pH. Remaining reagent was eliminated by passing the solutions over a Sephadex G50 column. Acetylated melittin migrated at about 1/3 of the distance of ordinary melittin in electrophoresis on cellogel strips at low pH. Succinylated melittin was run at pH 5 in an acetic acid/ammonia mixture and migrated to the anode, consistent with an expected net charge number of $-2$. In agreement with results found by others [17] it gave a smeared spot, probably due to aggregation, which persisted in the presence of urea. Nevertheless, full succinylation was indicated by the absence of a ninhydrin reaction. Furthermore, homogeneity of the modified peptides was demonstrated by HPLC.

Tryptophan modifications by *N*-bromosuccinimide (NBS) and 2-hydroxy-5-nitrobenzylbromide (HNBB) were performed as described by Talbot et al. [12] and Vogel and Jähnig [16], respectively. Excess reagent was removed by ultrafiltration over a Diaflo YC05 membrane.

### *Methods*

Water-membrane partitioning was determined by titrating various concentrations of peptide with small unilamellar DOPC vesicles (titanium tip sonicated at 10°C) and observing the changes in ellipticity at 222 nm. Experiments were performed at 20°C, typically in

TABLE I

*Aggregation constants of melittin and its derivatives*

All values apply to pH 7, if not otherwise stated. $K_4 = c_4/(c_1)^4$, $K_2 = c_2/(c_1)^2$ where $c_i$ is the concentration of an i-mer. For $K_2$ only rough estimates are given (cf. Materials and Methods), 'Very large' means that essentially all material is in aggregate form already at concentrations around 0.5 $\mu$M.

| Melittin derivative | $4 \cdot K_4$ ($M^{-3}$) | $2 \cdot K_2$ ($M^{-1}$) | Condition |
|---|---|---|---|
| Unmodified | $4 \cdot 10^{10}$ | | 0.1 M NaCl |
| | $3 \cdot 10^{16}$ | | 1 M NaCl |
| | $3 \cdot 10^{19}$ | | 1.8 M NaCl |
| | $10^{20}$ | | 1.8 M NaCl, pH 8.2 |
| Formylated | $5 \cdot 10^{16}$ | | 1 M NaCl |
| Acetylated | $2 \cdot 10^{16}$ | $2 \cdot 10^4$ | 0.1 M NaCl |
| | $5 \cdot 10^{19}$ | $3 \cdot 10^6$ | 1 M NaCl |
| | very large ($> 10^{23}$) | | 1.8 M NaCl |
| Succinylated | $7 \cdot 10^{16}$ | $4 \cdot 10^4$ | 0.1 M NaCl |
| | very large ($> 10^{23}$) | | 1 M NaCl |

10 mM Tris-HCl buffer (pH 7) with added 1 mM EDTA and 0.1 M NaCl, unless otherwise stated explicitly in the text. The ellipticity signal $F_\infty$ corresponding to fully vesicle-associated peptide was determined by extrapolation [18,19]. This $F_\infty$ was used to calculate the molar ratio of peptide associated per lipid, $r$, as a function of the free monomeric peptide concentration in solution, $c_1$. We will refer to the $r$ versus $c_1$ plots as 'isotherms'. Correction for tetramerization in aqueous solution was done as described previously [19]. Where necessary, the correction procedure was extended to include dimerization.

Aggregation constants for ordinary melittin and its derivatives at various salt concentrations were obtained as in Ref. 20 either from the wavelength shift of the emission peak of tryptophan fluorescence or from the change in ellipticity which accompanies the aggregate formation. They are compiled in Table I. In the case of the modified melittin derivatives, it is not clear whether they exist in a monomer-tetramer equilibrium like ordinary melittin or whether dimers and higher oligomers also appear in significant amounts. The presence of octamers was demonstrated by analytical ultracentrifugation for succinylated melittin at high salt concentration (2 M NaCl, 20 $\mu$M succinylated melittin, sedimentation equilibrium runs at 28000 and 44000 rpm: apparent molecular mass was about 25 kDa). However, under the conditions of our water-membrane partition experiments (0.1 M NaCl, peptide concentrations below 10 $\mu$M) octamer contributions appeared to be negligible (in sedimentation equilibrium runs at 0.1 M NaCl the main contribution was tetrameric, 11 kDa, even with 30 $\mu$M peptide). It was more difficult to assess whether dimers were present or not, since their contribution is most important at low peptide concentrations where the precision of spectroscopic measurements was comparatively low. We tried to fit the shift in peak wavelength of the fluorescence emission (or change in ellipticity at 222 nm) as a function of the total peptide concentration in aqueous solution, assuming that the dimers and the tetramers contribute equally (on a per residue basis) to the optical signal. This idea implies dimers of modified melittin to be assembled in a similar way as those which combine to form the tetrameric structure of ordinary melittin [4,20]. The resulting dimerization ($K_2$) and tetramerization ($K_4$) constants are given in Table I. Due to the uncertainties and assumptions mentioned above, the dimerization constants must be considered as rough estimates. This is, however, not critical to the conclusions drawn in the context of this article, since aggregation corrections mainly affect the high concentration part of the isotherms, where we limit ourselves to qualitative conclusions (e.g., comparing the strong rise of the isotherms with DMPC to the absence of this phenomenon with DOPC). For the succinylated melittin isotherm in Fig. 1 changes in the aggregation parameters would mainly result in a horizontal shift of those points which already lie in the flat branch of the isotherm, leaving the overall shape of the curve intact.

At low concentrations (smaller than about 1 $\mu$M) the corrections are in any event small, and the choice of the particular model used for calculating them is not critical. In fact, this low concentration range is of interest for vesicle permeabilization and BLM conductance studies. Moreover, electrostatic repulsion between associated melittin molecules can be neglected here. Accordingly ideal partitioning of the peptide between the lipid and the water moieties applies as described by

$$r = \Gamma \cdot c_1 \qquad (1)$$

with an appropriate partition coefficient, $\Gamma$, based on the above defined concentration variables $r$ and $c_1$ [13]. This $c_1$ is the thermodynamically relevant aqueous peptide concentration rather than the total peptide concentration in the system.

Conductance experiments on planar DOPC bilayers were performed on bilayers painted from lipid solutions in decane in the same way as described previously [10]. The solvent-containing membranes obtained in this way have the distinct advantage of high mechanical stability. This property allows one to use extensive stirring, which is needed to obtain full equilibration of the highly water-soluble peptides with respect to their partitioning between the membrane and the bathing solution.

For marker release experiments, vesicles were prepared by sonication of POPC in the presence of 50 mM

5(6)-carboxyfluorescein (Sigma) in buffer and were separated from the external (dye containing) solution over a Sephadex G50 column. These vesicles were added to an equilibrated peptide solution at 20°C. Dye efflux was monitored from the increase of fluorescence at 525 nm as a function of time (excitation at 490 nm). 100% release was determined after having added a sufficient amount of Triton X-100 (see Ref. 21 for more details). We added vesicles to dissolved peptide in order to avoid artifacts arising from local concentration inhomogeneities that could result if the peptide was injected directly from the concentrated stock solution. All fluorescence experiments were done on a Schoeffel RS1000 fluorometer. As a measure of the (initial) rate of bilayer permeabilization (possibly due to pore formation) which is induced by the peptide, we determined an apparent index number of permeabilization in the course of the first 50 s, namely

$$p_{50} = -\ln\{(F_\infty - F_{50})/(F_\infty - F_0)\} \quad (2)$$

($F_0$, $F_{50}$, $F_\infty$ being the fluorescence emission intensities at time $t = 0$, 50 s and after total release, respectively). Assuming sufficiently long-lived pores, this number is equal to the average number of pores that have been formed per vesicle in the given period of time [21]. The measurements were done for various different concentrations of the total peptide while keeping the lipid concentration invariant.

## Results

We describe here the experimental results for each compound, relating them to the respective results obtained with unmodified melittin. Results bearing on solution aggregation, partitioning, and pore-formation reactions are condensed in Table II and referred to in Discussion.

### *Formylated melittin*

A minor fraction of natural bee venom, this compound is formylated at the N-terminal Gly, which eliminates the basic $\alpha$-amino group there. It has previously been shown to associate with DMPC bilayers at neutral pH in much the same way as unformylated melittin associates at pH 8 where the N-terminal is essentially neutral [19]. In particular, neither changing the pH nor formylation had an effect on the partition coefficient. The same was observed when we repeated these experiments with DOPC vesicles instead of DMPC (Fig. 1). However, tetramer formation in aqueous solution is promoted by eliminating the N-terminal charge as shown in Table I.

Conductance experiments (at pH 7) with the same material in planar DOPC bilayers gave the following results. The slow approach towards a stationary state after addition of peptide was faster than for ordinary melittin (10–15 min vs. 50 min, see Fig. 2). There was no change in the dependence on concentration (third to fourth power of monomer concentration) or on voltage (22 mV for an $e$-fold conductance increase, see Fig. 3). Current-voltage curves were displaced to higher voltages by some 10 mV as compared with ordinary melittin at neutral pH, reflecting the increased aggregation of the formylated compound in aqueous solution. All of these effects at pH 7 exactly reproduce the results obtained with ordinary melittin at pH 8.2 [10].

TABLE II

*Comparison of aggregation in aqueous solution, water-membrane partitioning and pore activity for different melittin modifications (at pH 7)*

| Melittin derivative | Aggregation [a] | $\Gamma$ [b] ($M^{-1}$) | Critical concentration ratio [c] | Apparent gating charge number [d] | Power law of pore activity [e] |
|---|---|---|---|---|---|
| Unmodified | 0 (2) | $4 \cdot 10^4$ | | 1.2 | 3–4 (2.5) |
| Formylated | 0 (4) | $4 \cdot 10^4$ | 1 | 1.2 | 3–4 |
| Acetylated | 2 (60) | $4 \cdot 10^4$ | 1 | 2.2 | 8 |
| Succinylated | 17 (100) | $4 \cdot 10^4$ | 10 | 2 | (~2.5) |
| Trp-modified | 0 (0) | $2 \cdot 10^3$ | 20 | n.d. | (~2.5) |

[a] Percent aggregated material at 1 $\mu$M total peptide for 0.1 M NaCl (in parentheses for 1 M NaCl).

[b] With 0.1 M NaCl and DOPC vesicles; for Trp-modified melittin the value is taken from Ref. 12 (see text) under conditions where $\Gamma = 4 \cdot 10^4$ $M^{-1}$ for unmodified melittin.

[c] Ratio of aqueous monomer concentrations of modified over unmodified melittin being equivalent regarding BLM conductance (formylated, acetylated) or marker release (succinylated, Trp-modified), respectively.

[d] Number of elementary charges formally transported across the bilayer upon the gating event ($= 26$ mV$/V_e$, where $V_e$ is the step in voltage needed for $e$-fold increase in conductance).

[e] Power of aqueous monomer concentration dependence for voltage sensitive conductance or (in parentheses) marker release rate.

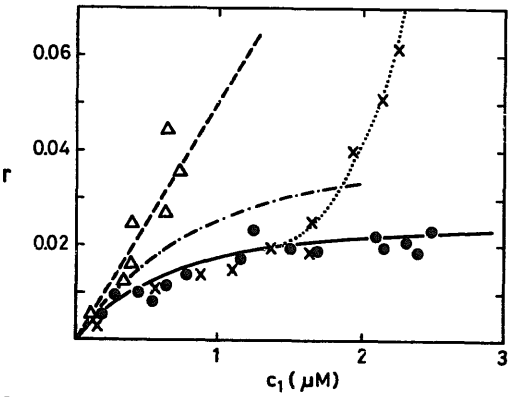


Fig. 1. Vesicle-associated peptide per lipid, as a function of aqueous monomeric peptide concentration; DOPC, 20°C, 0.1 M NaCl, pH 7. Natural melittin: continuous line; formylated melittin: dashed-dotted line; acetylated melittin: dashed line and triangles; succinylated melittin: full points. Crosses with dotted line: succinylated melittin with DMPC (at 30°C, 0.1 M NaCl, pH 7).

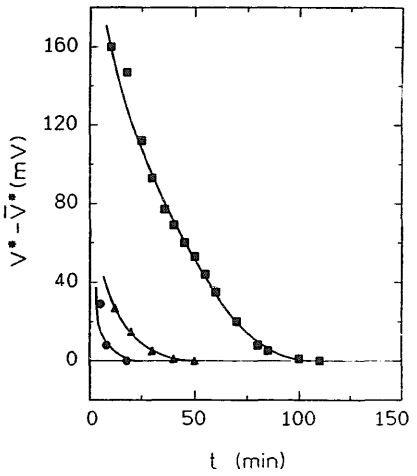


Fig. 2. Slow time course of the characteristic voltage at a conductance of 30 nS (V*) to the stationary state (V*) for unmodified (▲), formylated (●) and acetylated melittin (■) at 1.8 M NaCl (pH 7); the total melittin concentration was 0.4 μg/ml in all cases. The lipid is DOPC.

*Acetylated melittin*

Acetylation neutralizes all charged groups except the two arginines near the C-terminus. The coefficient for the partitioning between water and DOPC bilayers was virtually the same as that of natural melittin, in agreement with results previously obtained with DMPC vesicles [19]. The isotherm remained nearly straight, indicating a surprisingly small contribution of the arginines to charging the bilayer surface (Fig. 1).

Due to decreased charge repulsion, aggregation in aqueous solution is much stronger with acetylated than with natural melittin (cf. Table I). These equilibria were still salt dependent, although less so than with ordinary melittin. At 1.8 M NaCl no significant amount

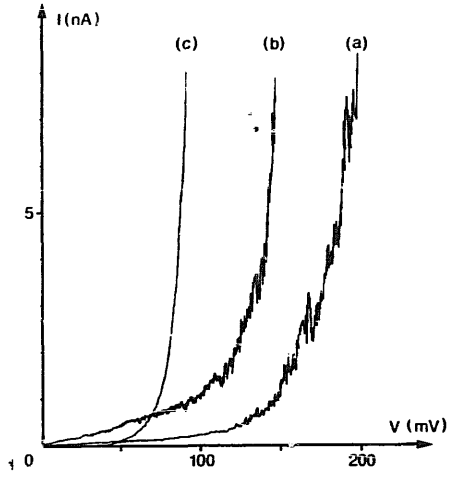


Fig. 3. Typical $I-V$-curves for (a) formylated (equivalent to unmodified) (at 1.8 M NaCl), (b) succinylated (at 1 M NaCl) and (c) acetylated melittin (at 1.8 M NaCl) on DOPC bilayers. The voltage step for an $e$-fold increase of the conductance ($V_e$) was (a) 22 mV, (b) 13 mV and (c) 11 mV. The positions of the $I-V$-curves along the voltage axis cannot be compared, because of different experimental conditions.

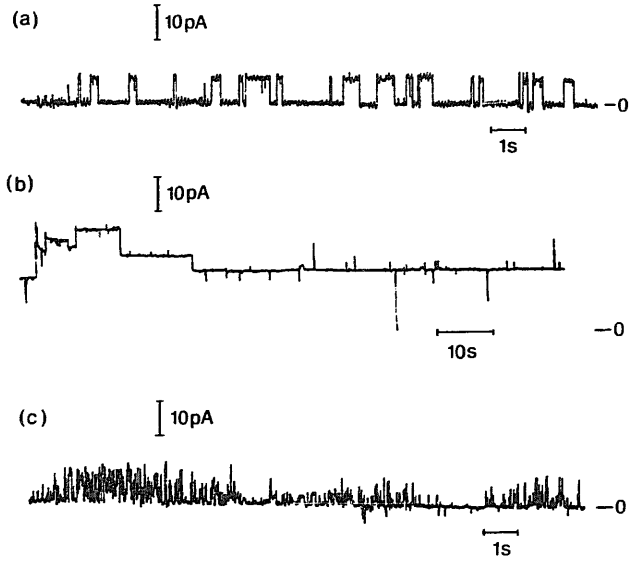


Fig. 4. Representative single pore traces with DOPC membranes. The peptide is (a) unmodified (at 1.8 M NaCl, 0.04 μM (peptide *cis* and *trans*), V = +70 mV), (b) acetylated (at 1.8 M NaCl, 0.27 μM (peptide *cis*), V = +50 mV) and (c) succinylated melittin (at 1 M NaCl, 0.12 μM (peptide *cis*), V = +50 mV).

of monomer could be detected at the lowest peptide concentrations amenable to spectroscopic analysis (about 0.2 μM). Nevertheless, conductance on planar bilayers could be readily observed under these conditions, although the time required for reaching the stationary state increased to about 2 h. The current-voltage curves were smoother than those obtained with normal melittin (Fig. 3). They showed twice as high a voltage dependence (11 mV per $e$-fold change in conductance, corresponding to a formal gating charge of two electron charge units per pore aggregate). Interestingly, the conspicuous asymmetry of the $I-V$ curves seen with unmodified melittin was retained: 80 mV higher '*trans*-positive' voltage (positive in the compartment without added melittin) was required to induce the same conductance as *cis*-positive voltage. In single-pore experiments very long-lived, stable pores could often be observed (Fig. 4).

Qualitatively the same results were obtained at 1 M NaCl, where the aggregation constants were measurable and thus the conductance results could be related to a defined aqueous monomer concentration. The conductance increased with about the 8th power of the free monomer concentration, instead of the 4th power obtained with unmodified melittin. The amount of aqueous monomer required to induce a given conductance was roughly the same as with ordinary melittin (column 3 of Table II; the uncertainty about the dimerization mentioned in Materials and Methods limits the accuracy of this numerical value to about a factor of 2).

*Succinylated melittin*

Succinylation also leaves the two positively-charged arginine residues unaltered, but introduces four nega-

tive charges in place of $\alpha$- and $\epsilon$-amino groups. Since melittin tetramers in aqueous solution consist of two antiparallel dimers associated one across the other [4], aggregation in solution is thought to be strongly promoted by the succinylation, which would bring negative groups from the N-terminal modified residues into proximity with the (unmodified) positive charges on the arginines at the C-terminal end. This expectation is borne out in our experiments: succinylated melittin was found to aggregate much more strongly than unmodified melittin even at low salt concentrations. Ultracentrifuge experiments (data not shown) indicated the presence of octamers at higher concentrations. In the concentration range up to 10 $\mu$M total concentration, the ellipticity changes could be fit with a model incorporating dimerization together with tetramerization (cf. Table I).

The coefficient for succinylated melittin partitioning between water and DOPC (20°C, pH 7, 0.11 M ionic strength solution) was $4 \cdot 10^4$ $M^{-1}$, which is the same as that obtained for unmodified melittin. With DMPC, succinylated melittin had a very strong tendency to break up lipid vesicles, as determined from a decrease in turbidity even at a free concentration of 2 $\mu$M. This was accompanied by a dramatic increase in ellipticity, as can be seen in Fig. 1. Such a behavior could also indicate aggregate formation in the bilayer phase in a similar way as has been reported for the peptide alamethicin [18].

Succinylated melittin was difficult to investigate with planar bilayers. Strong solution aggregation reduces the number of monomers available for partitioning, and the compound shows an even greater tendency to break bilayers than melittin itself. Single pore events that were obtained were rather noisy (Fig. 4). Current–voltage curves were steeper than those obtained with ordinary melittin but similar to the acetylated melittin results, with about 13 mV required per $e$-fold conductance increase (Fig. 3). The difficulties encountered prevented us from a systematic study of the concentration dependence.

We performed dye-efflux measurements with POPC vesicles in part because of the difficulties with the voltage-dependent conductance measurements. Under the conditions of the assay (20°C, 0.11 M ionic strength, pH 7), all compounds we tested were capable of releasing the dye from the vesicles, with unmodified melittin being the most efficient. The apparent efflux rate was quantified in terms of $p_{50}$ as defined by Eqn. 2. Fig. 5 shows plots of the log of this quantity plotted vs. the free monomer concentration (this is virtually equal to the total aqueous peptide concentration at these concentrations, but for succinylated melittin a small correction for aggregation was made).

The slope of any of the plots indicates an order of about 2.5 for the rate determining step of pore-formation under these conditions, for both the succinylated and unmodified melittin as indicated by the line drawn through the points. The concentrations of the modified compound required to reach a certain level of efflux were about 10-fold higher than the equivalent concentrations for normal melittin. Since the plot is already corrected for the aggregation reactions, and the partition coefficient is essentially unaffected by the modification, we conclude that the pore-forming ability of this compound in the membrane is reduced compared to that of unmodified melittin.

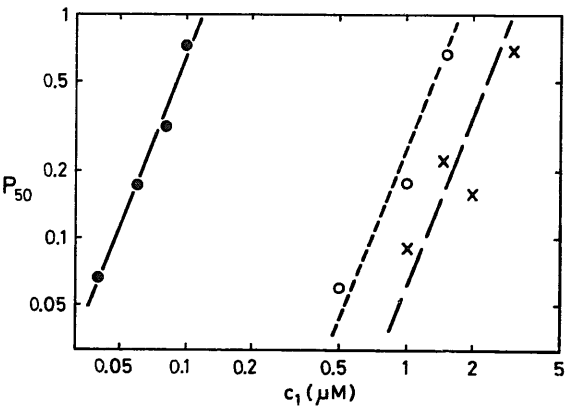


Fig. 5. Initial rate of marker release from POPC vesicles (0.1 M NaCl, pH 7) in terms of $p_{50}$ (see Eqn. 2) as a function of aqueous monomeric peptide concentration. Unmodified melittin: full points; succinylated melittin: open points; trp-modified melittin: crosses.

*Trp-modified melittin*

We used NBS induced oxidation of the tryptophan in position 19, eliminating the aromaticity of the ring. This modification results in a decreased tendency of the peptide to aggregate in aqueous solution. Under the conditions of the present study, the compound remained nearly fully monomeric at all concentrations in the aqueous phase. Melittin modified in this way has been shown to have a lower lipid affinity than unmodified melittin by Talbot et al. [12]. Conversion of their results (Fig. 5 of that reference) into an association isotherm using the techniques explained in Ref. 18, gives odd results for the modified melittin, but the partition coefficient can be judged to be at least one order of magnitude smaller than for normal melittin. For the latter we obtain a value of $4 \cdot 10^4$ $M^{-1}$ from the same data, in accord with our values for DOPC and those of others [13].

Marker efflux experiments with the Trp-modified melittin showed again an efficiency about 8 to 10-fold worse than that of unmodified melittin as can be seen in Fig. 5. The reduced partitioning essentially accounts for this drop in efficiency. In spite of the quite noticeable scattering of the data (see crosses in Fig. 5) it appears that the concentration dependence has about

the same power law as encountered for natural melittin.

*Combination studies*

We also examined mixtures of native, succinylated and tryptophan-modified melittin by making use of the dye efflux technique. At very small, but fixed concentrations of native melittin, addition of the modified peptides tended to decrease the efficiency of marker efflux. The decrease in the apparent efflux rate was very slight for concentrations of natural melittin of 0.05 $\mu$M and below, but became more and more pronounced when these concentrations were raised.

## Discussion

To facilitate the presentation of the results, Table II is organized into five major columns to indicate the relative changes resulting from each of these modifications on the aggregation in solution, the partitioning and the pore-formation behavior of the derivatives.

*Aggregation in solution*

Those melittin derivatives where charges have been modified behave largely in the expected way: a decrease in the total charge on the molecule results in an increase in the aqueous aggregation at a given salt concentration. A relatively close distance between certain positively charged residues in the aggregate can be inferred from the obvious successive increase of aggregation in the order native < formylated < acetylated < succinylated (cf. Ref. 22). The fact that the effect exists even at 1 M salt (with a Debye length of 3 Å) suggests that the aggregate involves a preferred location of positive charges relatively close to each other which must be stabilized by some other, presumably hydrophobic, interaction. Succinylation, where charge repulsion is replaced by attraction, then greatly promotes aggregate stability that persists even at low salt content.

The tryptophan-modified melittin shows a decreased aggregation in solution, as judged from the higher concentrations needed to reach moderate helicity in the CD spectrum when compared to normal melittin. One would imagine the NBS oxidation used in our studies to be a more conservative change from the modification procedure utilizing HNBB [16,17]. The latter procedure adds several carbons to the perimeter of the indole ring, leading to increased aggregation as compared to unmodified melittin (fluorescence assay; data not shown). Nevertheless, the tryptophan appears to be of critical importance to the behavior of the molecule, since the NBS modification dramatically decreases the solution aggregation properties and the bilayer affinity. The importance of the tryptophan for aggregation in solution had previously been stressed in permutation and replacement studies using synthesized peptides [23].

*Water-membrane partitioning*

The partitioning of all compounds was studied by titration of peptide solutions with lipid vesicles and analyzing the resulting association isotherm obtained by methods presented elsewhere [13,18,19]. The resulting partition coefficients relative to unmodified melittin under similar conditions are listed in Table II. The partition coefficient actually describes the initial linear part of the isotherm, which is the region of principal importance at the low levels of association typically used in pore-formation studies. At higher concentrations, isotherms flatten out due to electrostatic repulsion as the bilayer is increasingly charged by adsorbed peptide molecules. A thorough investigation of this part of the isotherm for natural melittin and some of the derivatives used in this study has been reported earlier [19]. Interestingly, within the uncertainties of our analysis mentioned in Materials and Methods, the isotherm of the succinylated compound exactly matches the one of natural melittin, though the latter has a net charge number of +6, the former only of −2. This result is consistent with the previous argumentation that the two arginines do not contribute very much to the effective interfacial charge [19]. Leaving out the arginines, the remaining charges are of opposite polarity but have the same absolute values for melittin as well as for its succinylated derivative.

At concentrations of several micromolar melittin is known to disrupt lipid vesicles. Succinylated melittin was found to be particularly effective in this respect with DMPC vesicles above the phase transition (30°C, see Fig. 1).

Combining these results, it appears that partition coefficients are practically insensitive to charge modifications on the melittin molecule. Such a conclusion agrees with that of a previous study on the pH dependence of partitioning [19]. On the other hand, however, the tryptophan residue seems to be an important modulator of membrane affinity. Modification of this residue decreases membrane affinity for the peptide by about one order of magnitude. This observation is consistent with at least a partial burying of the tryptophan residue in the nonpolar part of the bilayer, in agreement with spectroscopic evidence [3,24,25].

*Pore formation*

*Concentration dependence.* Remarkably, all modifications investigated showed some pore activity or permeabilizing power, either with BLM or in vesicles. This is true even for the succinylated molecule, which had been reported not to be hemolytic in erythrocytes [17,26]. Repulsion between the negatively charged succinyl groups and the charged sugar or protein moieties

on the erythrocyte surface could possibly account for such a protective effect.

Succinylated and Trp-modified melittin both needed about 10-fold higher concentrations in order to induce the same amount of dye leakage from vesicles as unmodified melittin. The reason for this, however, is probably different for the two compounds. In the case of the tryptophan modification, reduced bilayer affinity is sufficient to explain the effect: about an order of magnitude higher aqueous concentrations are needed to yield the same amount of peptide associated with the bilayer. In contrast, membrane partitioning of succinylated melittin is the same as that of unmodified melittin, therefore, this cannot be the reason for the decreased pore activity of the derivative. These observations are particularly surprising in the light of the observations that succinylated melittin is quite effective with regard to disrupting small DMPC vesicles at concentrations as low as 2 $\mu$M. Likewise it showed a strong tendency to break the bilayer in BLM experiments, and conductance traces at fixed voltage were very noisy. Taken together, these results suggest that succinylated melittin has stronger lipid-perturbing or bilayer-disrupting tendency than normal melittin, but pores made from this compound are less probable or are poorly conducting. With this derivative one therefore encounters an interesting dissociation of the two effects, bilayer disruption and pore formation ability. Conceivably, the pore-aggregate is formed as with normal melittin but the bulky succinyl groups somehow block the pore, perhaps by association with the remaining arginine residues.

The present findings clearly demonstrate the importance of complementing conductance or efflux experiments with measurements of thermodynamic properties such as water-membrane partitioning as well as aggregation in the aqueous phase (and any other effects that modulate the external monomer concentration). Only in this way the fundamental difference between Trp-modified and succinylated melittin showed up, whereas consideration of the efflux data alone (Fig. 5) could easily mislead.

Natural melittin as well as the two derivatives tested by the dye efflux assay all had about the same 2.5 power dependence on aqueous peptide concentrations (cf. Fig. 5). In contrast, more than twice as large a power dependence was obtained for acetylated than for ordinary melittin in BLM conductance experiments (where the latter has a 3rd to 4th power dependence) [2,10]. When comparing results from both sources it must be kept in mind that the physical basis of the effects measured is quite different for the two techniques. In marker release we deal with a kinetic phenomenon reflecting primarily the pore formation gross rate so that the apparent power law is determined by some rate limiting aggregation step in the over-all reaction process [21]. On the other hand, the conductance measures the net number of existing pores. If these are subject to a thermodynamic equilibrium the power law can be directly interpreted in terms of the molecularity of the pore. However, one may as well deal with steady state conditions implying a power dependence which is related to the number of monomers in an intermediate aggregate [10]. Thus, the much enhanced concentration dependence in the case of acetylated melittin does not necessarily reflect a larger size of the pore complex but might as well arise from a change in the kinetic scheme of pore formation (compared to unmodified melittin). The latter point would also be in line with the fact that the approach towards the stationary state of conductance on BLM is appreciably slowed down when using acetylated melittin. In any event, a higher power concentration dependence of dye efflux as well as voltage-dependent conductance clearly indicates crucial aggregation steps in the underlying molecular mechanism. This strongly suggests that all the observed permeabilizations are indeed caused by the formation of porous aggregates.

*Charge effects.* In agreement with previous studies [8,9], elimination of charges, especially in the N-terminal half of the peptide (formylated and acetylated melittin), does not abolish its pore activity. This result excludes the hypothesis [7] that the transbilayer voltage acts on the N-terminal charges to open the pore. In fact, the conductance response to an applied voltage step has been shown to be very fast, in the submillisecond range [10], which also stands against a model where the electric field pulls the peptide across the bilayer. Furthermore, if the peptide dipole (corresponding to about one half 'gating charge') and the N-terminal charge were to be pushed all the way across the bilayer, and lysine-7 at least half way across in a voltage-dependent manner, the formal gating charge expected would be about two electronic units per monomer (eight for a hypothetical tetrameric pore), which is much higher than actually observed. We therefore argue that the basic membrane spanning conformation is established in a slow step before pores can be readily activated by applying the electric potential. Such a view is in line with the observed waiting time needed for stable pore activity to develop. Significantly, that waiting time is decreased if the N-terminal charge is blocked (formylated melittin), thereby confirming conclusions drawn from experiments conducted at pH 8.2, where the N-terminus is expected to be deprotonated [10]. The acetylated derivative, however, behaves differently (cf. above).

The apparent existence of a pore-related transmembrane conformation of melittin even in the absence of voltage does not contradict spectroscopic evidence in favor of a state essentially associated with the membrane surface [5,6]. In fact, all the relevant 'prepore'

material may comprise only a very minor part of the total bilayer-adsorbed peptide.

Whereas the N-terminal charge of melittin appears to be involved in a 'prepore' state, the other charged groups seem to be important in voltage-dependent steps. Voltage dependence of the macroscopic conductance increases with acetylation or succinylation ($e$-fold change every 11–13 mV instead of 22 mV for melittin and formylated melittin). The underlying molecular mechanism is difficult to guess, even more so since we found that ordinary melittin shows a similar increase of voltage dependence ($e$-fold change every 12 mV) when studied in diphytanoyl-PC membranes instead of DOPC.

With acetylated melittin, single-pore behavior changes dramatically, and very long-lived pores show up. Apparently, absence of charge stabilizes the pore state.

In the most popular model of a pore complex formed from a bundle of parallel $\alpha$-helices [8,12,16], repulsion of like charges on opposing peptide strands may be expected to destabilize the pore aggregate. We therefore wanted to check whether pores were stabilized upon mixing peptides bearing complementary positive and negative charges, respectively, i.e., natural and succinylated melittin. This expectation was not borne out: in marker release studies, addition of succinylated to natural melittin decreased the pore activity. It is possible that the decrease was due to the formation of mixed aggregates in the aqueous solution thereby reducing the pool of free available melittin monomers. Such a view is supported by the experiment shown in Fig. 6. Titrating succinylated melittin in the presence of a fixed concentration of natural melittin resulted in much higher ellipticities than the two compounds exhibit together when taken separately. Other effects may as well contribute to the observed decrease of the efflux rate, e.g., formation of mixed pores which are

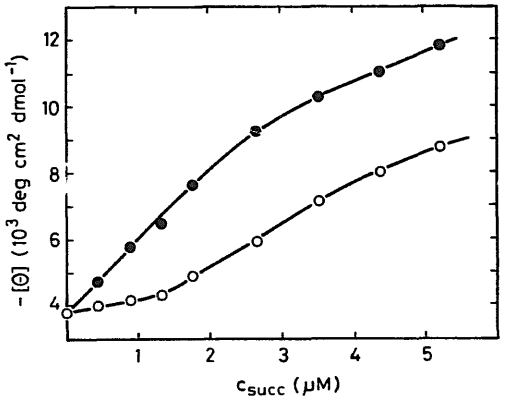


Fig. 6. Ellipticity of a mixture of 2.5 $\mu$M unmodified melittin and succinylated melittin, as a function of total concentration of the latter (full points). Open points indicate theoretical values obtained by adding individual ellipticities of both components at the same concentrations.

poorly conductive, perhaps because of being blocked by residues forming salt bridges.

Despite the wealth of data accumulated so far, there is still no clear picture of the molecular mechanism of melittin induced pore formation. Nevertheless, with the information now available one can rule out some too simple models and develop more realistic ones. Our results should also prove useful in guiding the choice of suitable residues for the synthesis of membrane-active peptides, an approach that is rapidly gaining importance in the study of channel proteins and toxins [9,21,27,28].

## Acknowledgements

We thank P. Savko and J. Wey for technical assistance, A. Lustig for performing the ultracentrifuge runs and evaluations and Dr. E. Kuchinka for a gift of formylated melittin. This work was supported by grant No. 31.25230.88 from the Swiss National Science Foundation.

## References

1 Dempsey, C.E. (1990) Biochim. Biophys. Acta 1031, 143–161.
2 Tosteson, M.T. and Tosteson, D.C. (1981) Biophys. J. 36, 109–116.
3 Vogel, H. (1981) FEBS Lett. 134, 37–42.
4 Terwilliger, T.C. and Eisenberg, D. (1982) J. Biol. Chem. 257, 6016–6022.
5 Stanislawski, S. and Rüterjans, H. (1987) Eur. Biophys. J. 15, 1–12.
6 Altenbach, C., Froncisz, W., Hyde, J.S. and Hubbell, W.L. (1989) Biophys. J. 56, 1183–1191.
7 Kempf, C., Klausner, R.D., Weinstein, J.N., Van Renswoude, J., Pincus, M. and Blumenthal, R. (1982) J. Biol. Chem. 257, 2469–2476.
8 Hanke, W., Methfessel, C., Wilmsen, H.U., Katz, E., Jung, G. and Boheim, G. (1983) Biochim. Biophys. Acta 727, 108–114.
9 Tosteson, M.T., Caporale, L.H. and Tosteson, D.C. (1988) Biophys. J. 53, 9a.
10 Pawlak, M., Stankowski, S. and Schwarz, G. (1991) Biochim. Biophys. Acta 1062, 94–102.
11 Hermetter, A. and Lakowicz, J.R. (1986) J. Biol. Chem. 261, 8243–8248.
12 Talbot, J.C., Faucon, J.F. and Dufourcq, J. (1989) Eur. Biophys. J. 15, 147–157.
13 Schwarz, G. and Beschiaschvili, G. (1989) Biochim. Biophys. Acta 979, 82–90.
14 Bradrick, T.D., Freire, E. and Georghiou, S. (1989) Biochim. Biophys. Acta 982, 94–102.
15 Kuchinka, E. and Seelig, J. (1989) Biochemistry 28, 4216–4221.
16 Vogel, H. and Jähnig, F. (1986) Biophys. J. 50, 573–582.
17 Habermann, E. and Kowallek, H. (1970) Hoppe Seyler's Z. Physiol. Chem. 351, 884–890.
18 Rizzo, V., Stankowski, S. and Schwarz, G. (1987) Biochemistry 26, 2751–2759.
19 Stankowski, S. and Schwarz, G. (1990) Biochim. Biophys. Acta 1025, 164–175.
20 Schwarz, G. and Beschiaschvili, G. (1988) Biochemistry 27, 7826–7831.
21 Schwarz, G. and Robert, C.H. (1990) Biophys. J. 58, 577–583.
22 Bello, J., Bello, H.R. and Granados, E. (1982) Biochemistry 21, 461–465.

23 Weaver, A.J., Kemple, M.D. and Prendergast, F.G. (1989) Biochemistry 28, 8624–8639.
24 Dufourcq, J. and Faucon, J.F. (1977) Biochim. Biophys. Acta 467, 1–11.
25 Georghiou, S., Thompson, M. and Mukhopadhyay, A.K. (1982) Biochim. Biophys. Acta 688, 441–452.
26 Dufton, M.J., Hider, R.C. and Cherry, R.J. (1984) Eur. Biophys. J. 11, 17–24.
27 De Grado, W.F., Kézdy, F.J. and Kaiser, E.T. (1981) J. Am. Chem. Soc. 103, 679–681.
28 Lear, J.D., Wassermann, Z.R. and De Grado, W.F. (1988) Science 240, 1177–1181.