Biochimica et Biophysica Acta, 1025 (1990) 164–172
Elsevier

BBAMEM 74893

Electrostatics of a peptide at a membrane/water interface. The pH dependence of melittin association with lipid vesicles

Stefan Stankowski and Gerhard Schwarz

Biocenter of the University of Basel, Basel (Switzerland)

(Received 6 December 1989)

Key words: Protein–lipid interaction; pH dependence; Melittin; Surface potential; Gouy-Chapman model

The association of the peptide melittin with small unilamellar DMPC vesicles was studied as a function of pH. The results are discussed quantitatively assuming a water-membrane partition equilibrium. Electrostatic surface charging is taken into account as more and more of the strongly basic peptide accumulates at the bilayer/water interface. The data could be well described in terms of a Gouy-Chapman approach involving an effective interfacial charge well below the actual physical charge carried by the individual peptide molecules. The partition coefficient turned out to be pH invariant, so that one can exclude deprotonation reactions upon insertion of the peptide into the bilayer. The effective interfacial charge per associated melittin molecule decreased over a broad range of pH (pH 7 to pH above 10). Contributions of the free amino terminus and of the arginine residues could be determined by comparing with results obtained using modified melittin (N-terminally formylated and fully acetylated). The data suggest approximately equal fractional contributions of the amino terminus and the three lysines to the effective interfacial charge. The two arginines contribute less. Thus, they may be located farther away from the interface or be closely associated with counter-ions. The analysis is extended to the effect of different ionic strengths.

Introduction

Melittin, a 26 amino acid peptide from bee venom, has aroused much interest in membrane research as a model for lipid–protein interaction [1], voltage-dependent pore formation [2], membrane fusion [3], induction of lipid polymorphism [4] and a wide spectrum of other biophysical, biochemical and pharmacological aspects. Nevertheless, the molecular details of its interaction with membranes are still a matter of much debate. Neither the exact conformation and location, nor the aggregation state in or at the membrane have been unequivocally determined. One particular aspect needing further clarification is the role of the various charged groups in the amino acid sequence, namely:

$$NH_3^+ \ldots \overset{7}{Lys}^+ \ldots \overset{21}{Lys}^+ \overset{22}{Arg}^+ \overset{23}{Lys}^+ \overset{24}{Arg}^+ \ldots CONH_2$$

Abbreviations: DMPC, dimyristoylphosphatidylcholine; DOPC, dioleoylphosphatidylcholine; CD, circular dichroism.

Correspondence: S. Stankowski, Biozentrum, Department Biophysical Chemistry, Klingelbergstrasse 70, CH-4056 Basel, Switzerland.

The pK values for these groups have been determined for the aqueous and bilayer-associated conformations of the peptide [5]. In natural bee venom a small fraction is formylated at the N-terminus.

New efforts have recently been made to evaluate membrane association of melittin in a quantitative way with explicit account of electrostatic effects produced by these charges [6]. Gouy-Chapman theory was found to be applicable, provided the melittin molecule is attributed an effective charge of the order of two elementary charge units (at pH 7) which is much lower than the actual physical charge. This is consistent with the influence of melittin on the lipid head groups, as assessed by ^{2}H-NMR [7,8] (see below for a more detailed discussion).

In order to gather more information about the location and electrostatic interactions of charged groups in membrane associated melittin we have measured association isotherms as a function of pH. These are processed into partition coefficients and effective interfacial charges under a variety of conditions.

Apart from effects of bilayer surface charging upon association of melittin, we are especially interested in the particular role of lysine 7. This basic residue lies in

the middle of an otherwise largely hydrophobic sequence (residues 1–19), which should have the capability of crossing a lipid bilayer, be it spontaneously [1], or be it under the influence of a transmembrane potential [9]. If membrane incorporation of this residue involved a deprotonation reaction, pH dependence should be a sensitive measure of such a process.

Materials and Methods

Materials

Melittin was purchased from Mack (Illertissen, F.R.G.) and purified by ultrafiltration through YM10 membranes (Amicon, Danvers, MA). Phospholipase A_2 contamination is removed by this procedure as checked by prolonged exposition of lipid to the filtered material, followed by thin-layer chromatography [6]. Electrophoresis on cellogel strips (Serva, Heidelberg, F.R.G.) in 4 M urea, 3 M formic acid [10] showed that this material contained an amount of N-terminally formylated melittin estimated to about 5%. This component can be separated by reverse phase HPLC [7]. Comparing isotherms obtained with the HPLC purified main component with those obtained with the natural mixture, we could not detect any significant differences, even at a pH below 7. Thus, the small amount of formylated melittin in the natural mixture does not affect the isotherms in a measurable way, and we used this material for the bulk of our experiments. Above pH 7, the amino terminus gets deprotonated, so that the two components should become indistinguishable for our purposes, anyhow. N-Terminally formylated melittin, separated by HPLC, was used in separate experiments. It was about 95% pure by electrophoresis.

Acetylated melittin was prepared as in Ref. 10. Its electrophoretic mobility was about one third of that of the main component of natural melittin, consistent with two charges (the two arginines) remaining out of six.

DMPC was partly from Fluka (Buchs, Switzerland), partly from Avanti (Pelham, AL), both batches giving identical results.

Methods

Small unilamellar vesicles were prepared by tip ultrasonication at 30 °C, followed by centrifugation to remove titanium particles. For experiments at pH above 8.5, vesicles were prepared at lower pH and titrated to the desired pH just before starting the experiments. No free fatty acids or lysophospholipids could be detected on heavily overloaded thin layer chromatograms of the sonicated material, which showed only one single spot.

Buffers (Hepes, Tris, diethanolamine) were prepared at room temperature. The pH values given in the figures and Table I are corrected for heating to 30 °C, as verified by direct measurement in the warmed cuvette. The buffer concentration was 10 mM throughout. EDTA was added (0.1 mM, but 1 mM for 1 M NaCl solutions). The NaCl concentration was adjusted to keep the ionic strength constant at different degrees of buffer dissociation. Thus, 0.11 M ionic strength means 10 mM Tris plus 0.1 M NaCl at pH 7 (where this buffer is largely dissociated), or 10 mM Tris plus 0.11 M NaCl at pH 9 (where the degree of dissociation is small).

TABLE I

Values of Γ and ν (see text) for melittin-DMPC association isotherms under various conditions

Temperature 30 °C. Typical uncertainty is 20% in Γ, ± 0.1 in ν. (Setting $a = 1$ in Eqn. 1 would decrease the given values of ν by about 0.1 throughout. Setting $\beta = 0.5$ would decrease ν by 5% whereas $\beta = 0.8$ (partial access to inner vesicle compartment) would increase ν by 10%).

Ionic strength	Melittin	Bulk pH	Γ [M^{-1}]	ν
0.11 M	natural	5, 6, 6.8	$1.8 \cdot 10^5$	1.9
	natural	7.8	$1.5 \cdot 10^5$	1.5
	natural	8.8	$1.5 \cdot 10^5$	1.3
	natural	9.6	$1.5 \cdot 10^5$	1.05
	natural	10.1	$1.5 \cdot 10^5$	0.8
	N-formylated	6.8	$1.5 \cdot 10^5$	1.5
	acetylated	6.8	$1.5 \cdot 10^5$	0.4
0.02 M	natural	6.8	$6 \cdot 10^4$	1.9
	natural	8.8	$6 \cdot 10^4$	1.05
1.01 M	natural	6.7	$1.5 \cdot 10^5$	0.3

Melittin association to DMPC vesicles was measured at 30 °C by adding vesicles to a fixed amount of peptide and monitoring the change of ellipticity at 222 nm on a Cary 61 circular dichrograph. The ellipticity value at 260 nm was also recorded as a base-line check, and the lipid contribution to the signal was subtracted after titration of vesicles in the absence of melittin, under otherwise identical conditions. Peptide concentrations were determined from the UV absorbance at 280 nm (absorption coefficient 5570 $M^{-1} \cdot cm^{-1}$). The ellipticity per residue of monomeric melittin in the absence of vesicles, θ_0, was found to be -3800 deg $\cdot$ $cm^2 \cdot dmol^{-1}$ at pH 7, decreasing to -5500 deg $\cdot$ $cm^2 \cdot dmol^{-1}$ at pH 10.5. Tetramerization constants of the aqueous peptide were determined from the concentration dependence of the CD signal, in analogy to the procedure described in Ref. 11. Corrections for tetramer formation in the aqueous solution were needed at very high salt concentration (1 M NaCl, pH 6: tetramer association constant, K_a is $1.3 \cdot 10^{16}$ M^{-3}) and for acetylated melittin (at pH 7, 0.1 M NaCl, $K_a = 5 \cdot 10^{15}$ M^{-3}). Under the remaining conditions, tetramerization was negligible at the melittin concentrations of 2–10 μM used. The correction procedure is given in Appendix 1.

The ellipticity of the fully vesicle-associated state, θ_∞, was determined from reciprocal plots and taking into account that isotherms must fall upon each other for experiments done at different peptide concentrations

under otherwise identical conditions (cf. Ref. 12 for details). The two-state assumption inherent in this procedure has previously been shown by a more general evaluation method to be valid for melittin with DOPC vesicles [6]. θ_∞ was about -19000 deg · cm^2 · dmol^{-1} for DMPC, decreasing to -21000 at high pH values and high salt concentrations.

All experiments were conducted at 30 °C, well above the phase transition temperature of pure DMPC (22–24 °C) where melittin is known to break up DMPC vesicles into smaller structures [13].

Melittin is known to disrupt vesicles also at temperatures above the phase transition at high peptide to lipid ratios and neutral pH [14]. We did two controls to check whether this occurred also for the low peptide concentrations used in the present study (2–9 μM) at the relatively large r values attained at high pH, high salt or with acetylated peptide. Negative stain electron microscopy showed vesicles of slightly increased size, and a few smaller structures, at peptide to lipid ratios corresponding to the final part of the isotherms of Fig. 1. In addition, we performed turbidity experiments (90 degree light scattering at 365 nm) of vesicle suspensions in the absence and presence of melittin. A strong drop of the turbidity was seen only at very high peptide to lipid ratios, corresponding approximately to the very last (upper or rightmost) points of the isotherms in Figs. 1 and 2 (the two upper points in Fig. 3). In the concentration range of the central to final part of the isotherms the turbidity was similar to or slightly larger than that of pure lipid vesicles. At higher excess of lipid (corresponding to the lower left part of the isotherms) the turbidity increased considerably in a slow time course, indicating melittin-induced fusion.

Thus, relatively high r-values could be reached under the conditions of this study (low concentrations, high pH or acetylated melittin, short incubation times) without significant vesicle disruption. Since our titrations were typically started at low lipid to peptide ratio, we furthermore verified that this did in any case not affect the results of the remaining titrations. Signals at higher lipid content were found to be independent of whether a large amount of lipid was added in one single step right from the start or in many consecutive steps.

Finally, it is good evidence for consistency that we find analogous trends as described below for DMPC also when using vesicles made out of DOPC (Avanti). The partition coefficient is lower for the unsaturated chain lipid than for DMPC. Consequently, the r values remain by about a factor of two smaller so that it should be safe to neglect vesicle disruption [14]. We preferred to do the present complete analysis with DMPC, however, because the risk of producing free fatty acids in the sonication procedure is larger for DOPC; the existence of negative charges in the bilayer would interfere with the electrostatic effects we want to evaluate. Parameters obtained with DOPC were: $\Gamma = (3–4) \cdot 10^4$ M^{-1}, independent of pH; $\nu = 1.8 \pm 0.1$ at pH 7 and $\nu = 1.1 \pm 0.2$ at pH 9.1 (fit ignores upward bending of the isotherm at high c_1).

Theoretical

Melittin association to phospholipid vesicles can be described by a simple model [6] incorporating a water-membrane partition equilibrium modulated by electrostatic charging of the (neutral) membrane as the basic peptide accumulates at the interface. The surface potential induced in this way counteracts the association of further peptide. It turns out that this effect can be satisfactorily treated using a Gouy-Chapman approach [6]. We use a slight extension, including a small correction to account for possible area expansion due to the insertion of the peptide into the bilayer. The surface charge density, σ, is set proportional to the surface density of associated melittin:

$$\sigma = \frac{\nu r/\beta}{1 + ar/\beta} \frac{e_0}{A_L} \tag{1}$$

Here, ν is the effective charge of a melittin molecule at the interface, in units of elementary charge, e_0. A_L is the surface area occupied by a lipid molecule in the outer leaflet of the bilayer.

$$r = c_a/c_L \tag{2}$$

is the concentration ratio of vesicle-associated peptide per total lipid. Since melittin is considered to have access only from the vesicle outside [5,6,8] r is corrected in Eqn. 1 by the fraction of lipid in the outer leaflet, β. Furthermore, the hydrophobic parts of melittin insert into the membrane. The corresponding expansion of the area is taken into account in Eqn. 1 by the denominator, where a is the ratio of effective molecular areas of melittin over lipid. This latter effect introduces only a small correction, and the chosen value of a is not critical.

Gouy-Chapman theory then relates σ to the surface potential, ψ [15]. The repulsive effect of the surface potential on the peptide can be formulated by means of an activity coefficient, α, which modulates water-membrane partitioning [6]:

$$\alpha r = \Gamma c_1 \tag{3}$$

Γ is the appropriate partition coefficient and c_1 the aqueous concentration of melittin monomers (note that tetramerization may occur in the aqueous solution). The activity coefficient can be calculated by standard methods [6]. It turns out that the formalism is equivalent to writing

$$r = \Gamma c_1 \exp(-\nu F\psi/RT) \tag{4}$$

with F the Faraday constant. Conceptually this can be interpreted as an ideal partition equilibrium where the bulk aqueous concentration of peptide is replaced by the concentration close to the interface, where a Boltzmann factor involving the surface potential is applicable.

Combining these equations the experimental isotherms can be fit with two free parameters: the partition coefficient, Γ (related to the initial slope of the curves) and the effective interfacial charge, ν (related to the degree of bending at high c_1 values). In all cases ν is found to be much smaller than the physical charge on the peptide [6]. The 'geometrical' parameters, a, β and A_L, have been considered previously and are taken over (with rounded values) from that work [6–8]: $A_L = 0.7$ nm^2, $\beta = 0.6$, $a = 2$. The exact choice of these parameters is not critical, as indicated in the legend to Table I. Letting β approach 0.5 in the lower left parts of the isotherms, where fusion tends to increase vesicle size, does not affect the fit parameters at all (there is good evidence that melittin has not access to the inner vesicular compartment in that domain of concentrations [5,10]). Increasing β in the upper or rightmost part of the isotherms to account for some possible vesicle disruption (in spite of the negative controls mentioned in Methods) giving the peptide access to the inner compartments, would increase ν by about 10%. Changes of this order can be tolerated without affecting the conclusions drawn below. Notably, the partition coefficient is independent of the particular choice of the 'geometrical parameters' which are only relevant to the activity coefficient.

Results

1. pH dependence of melittin association with DMPC vesicles

Association isotherms of melittin with DMPC vesicles (i.e., ratio of associated peptide to lipid, r, versus the concentration of monomeric peptide in the aqueous phase, c_1) have been measured for various values of bulk pH. The corresponding data (taken at 30 °C and at 0.11 M ionic strength) are shown in Fig. 1. Solid lines are drawn according to the theoretical model given above (Eqns. 1–4, see Ref. 6 for details) with the parameters listed in Table I. Parameters obtained with DOPC are given for comparison under Methods. Two conclusions are obvious from these figures:

(1) The initial slope of the isotherms is practically independent of pH. Accordingly the same partition coefficient Γ is obtained at all values of pH (Table I).

(2) The degree of bending towards the abscissa decreases substantially with increasing pH, indicating a decrease of the effective interfacial charge, ν. The relevant parameter values are given in Table I. Such a behavior is naturally expected due to the progressive

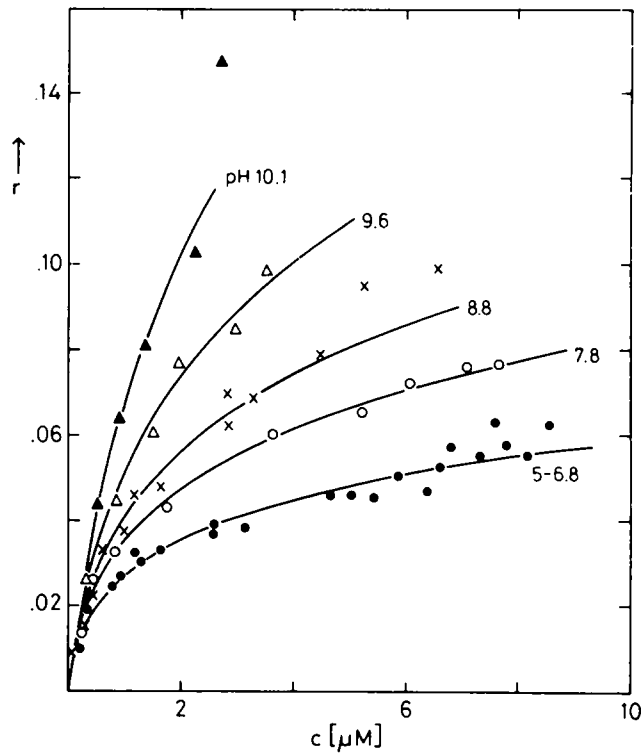

Fig. 1. Vesicle-associated peptide per lipid, r, versus the concentration of aqueous peptide monomers, c, at 30 °C at an ionic strength of 0.11 M, for various values of bulk pH. Solid lines are fitted using the parameter values listed in Table I. Peptide concentrations were: 8.25 μM at pH 5 and 6; 4.8 and 9 μM at pH 6.8; 8.8 μM at pH 7.8; 8.1 and 3.8 μM at pH 8.8; 4.3 μM at pH 9.6; 3.8 μM at pH 10.1.

loss of charges at elevated pH. However, the question arises how the effective charges derived from the isotherms relate to the physical side chain charges on the membrane-associated peptide. In principle, this question can be answered by comparing association isotherms of melittin where charged side chains are specifically replaced or blocked.

2. Comparison with modified melittin

Melittin with its amino terminus blocked by a formyl group is a minor component of bee venom. It can be separated chromatographically. Titrating this material with DMPC vesicles at a pH of 6.8 yielded the same isotherm as obtained with the non-formylated species at pH 7.8 (Fig. 2). Thus, the change in the isotherms of Fig. 1 observable between pH 6.8 and pH 7.8 can be unequivocally attributed to titration of the N-terminal amino group.

The role of the two arginines in positions 22 and 24 can equally be assessed using acetylation of melittin. By this procedure, the amino terminus and the three lysines become blocked, leaving the two arginines as the only charges. The corresponding isotherm at pH 6.8 is shown in Fig. 2. It is close to the pH 10.1 curve of natural melittin. Thus, the three lysines must be largely deprotonated at this value of bulk (!) pH. The effective

interfacial charge number derived for acetylated melittin is low, about 0.4, the exact value being uncertain for the following reasons: Firstly, this parameter is primarily determined from the degree of bending of the association curve. Bending being weak for the acetylated compound isotherm, very high values of r would be needed to define the parameter ν unambiguously. These high values cannot be obtained experimentally, because the vesicles would break up at such high peptide to lipid ratios. Secondly, the raw data had to be corrected for tetramerization in the aqueous solution, which is substantial for acetylated melittin even at 0.1 M NaCl. Errors in the parameters used for the corrections of course add up to those in the vesicle titration.

3. Variation of salt concentration

The experiments presented so far were all done at 0.1 M NaCl added to the buffer (plus a small additional amount to compensate for the variable degree of buffer dissociation at different pH, in order to keep the ionic strength constant). Some of the experiments were repeated in the presence of lower (10 mM) and higher (1 M) sodium chloride concentrations. The effective charge number, ν, was only slightly altered at low salt, but the partition coefficient decreased. The latter is in contrast to observations with DOPC vesicles [6]. At high salt, the partition coefficient remained virtually invariant whereas the effective charge decreased markedly. The respective parameter values are listed in Table I. Fig. 3 compares the experimental isotherm at 1.01 M ionic strength with the one obtained at 0.11 M ionic strength. Also shown is the change to be expected from increased ionic screening, as calculated from our Gouy-Chapman approach for a 1.01 M salt concentration, but with the same Γ and ν as for the 0.11 M isotherm. The experimental curve is clearly much less bent, demonstrating the lower value of the interfacial charge.

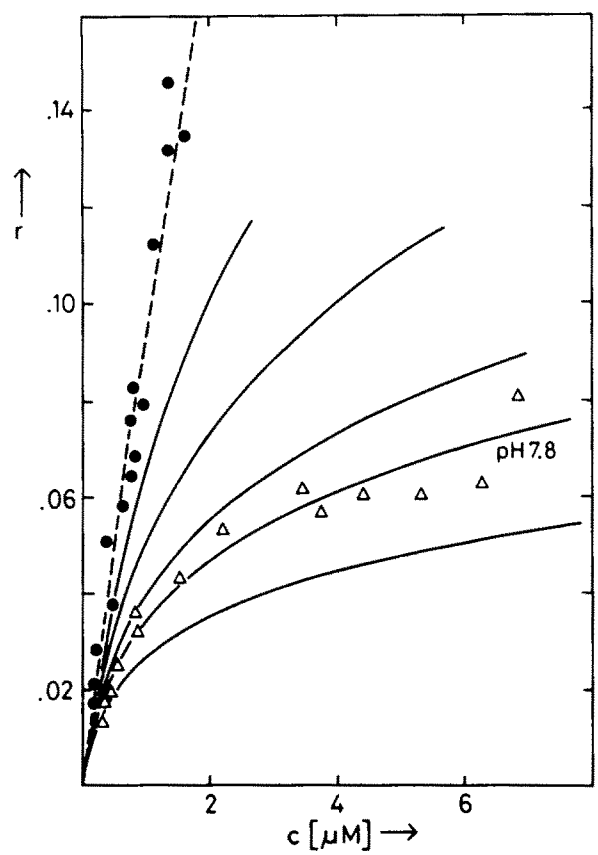

Fig. 2. Association isotherms for N-terminally formylated melittin, 6.3 and 8.1 μM (triangles) and acetylated melittin, 2 and 4.4 μM (full circles) at pH 6.8. Broken line is fitted with parameters given in Table I. Solid lines for the pH dependence of natural melittin are taken over from Fig. 1.

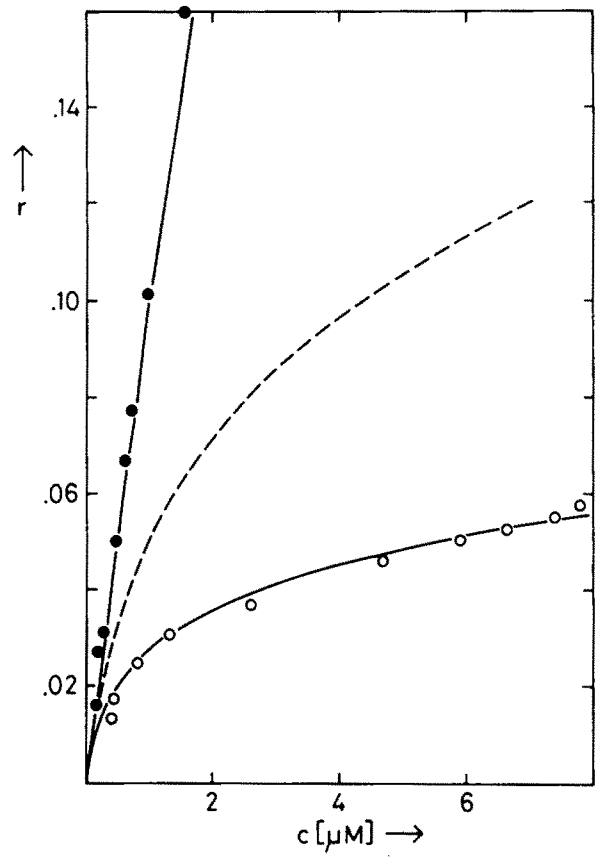

Fig. 3. Melittin association isotherms at pH 6 with 0.1 M NaCl (open circles, cf. Fig. 1) and 1 M NaCl (full circles 2.6 and 4.2 μM). Parameters of fits (solid lines) are given in Table I. The broken line is the isotherm calculated for 1 M NaCl concentration using the parameters of the 0.1 M NaCl curve.

Discussion

Measuring association isotherms of melittin with phospholipid vesicles such as those shown in Fig. 1, yields two basic properties: the partition coefficient (extrapolated from the initial slope of the curves) and the membrane surface potential as it changes upon peptide accumulation at the interface (derived from the

degree of bending of the curves; our results in fact prove the previously assumed electrostatic nature of this bending, since the effect diminishes as the charges disappear during pH titration). How these two features depend on pH can therefore be deduced from the present data.

For comparison with previous work, one should bear in mind that all information obtained here is for the membrane associated form of melittin at 30 °C (implying that the pK values of functional groups are slightly reduced with respect to 20 °C). pH values are given for the bulk solution. Build-up of a positive surface potential in the presence of melittin decreases the proton concentration at the interface. This interfacial pH is not constant, but increases in the course of an isotherm. In the bent part of our assoication curves (which determines the effective charge number ν) surface potentials typically reach values of the order of 35–40 mV. The pH at the bilayer interface would then be about 0.6–0.7 pH units larger than the bulk value. Surface potentials are smaller at large ionic strength (1.01 M), but the opposite is true for the 20 mM series. The difference between bulk and interfacial pH is particularly large in that case and may be sufficient to explain the slightly lower values of effective charge ν evaluated (cf. Table I). Furthermore, an increase of (local) pH may be the reason for a rising tendency at the very end of some isotherms (e.g., at pH 8.8).

Our isotherms at neutral pH look qualitatively similar to those obtained previously with DOPC and POPC [6,7]. The partition coefficient is somewhat larger for the saturated lipid. There are larger differences to published results obtained with DMPC vesicles at 25 °C [16]. We attribute these differences to the fact that the latter temperature is very close to the phase transition region of that lipid, where melittin is known to exert special effects [13].

pH invariance of the partition coefficient

Various models have been proposed for the insertion of melittin into phospholipid bilayers, some of which assume only partial insertion of hydrophobic residues into the outer leaflet of the bilayer [5,9,17,18], others a membrane spanning conformation of the peptide [10,19,20]. There is also evidence that melittin interacts differently with different kinds of lipids [4], and we would not exclude the possibility of different molecular states coexisting on one type of bilayer. There is, however, general consensus that melittin in its membrane-associated form adopts a structure characterized by two α-helical stretches interrupted by a bend around proline 14. The free energy gain from incorporating this part of the molecule (residues 1–20) into a lipid bilayer would be large enough to compensate for the deprotonation of the only charged residue in this stretch, Lys-7 (needing a free energy of 20 kJ/mol at pH 7). This is easily verified using tabulated values of transfer energies [21]. Clearly, a deprotonation reaction would be strongly pH dependent. Looking for such an effect had been our main motivation to start the present investigation.

To our surprise, the water-membrane partition coefficient turned out to be insensitive to pH. Thus, models involving insertion into the bilayer with concomitant deprotonation of Lys-7 can be ruled out. Qualitatively, we found the same results for DOPC instead of DMPC. Therefore the conclusion is not limited to saturated chain lipids but remains valid for a broad class of phosphatidylcholines.

The observed pH invariance of the partition coefficient is surprising also in another respect. According to physical principles, free energy is even needed to bring a charge from the bulk aqueous solution close to a membrane interface even if it is not inserted into the hydrocarbon core. Elementary electrostatic calculations (Schwarz, G., unpublished data) yield estimates of the order of 8.5 kJ/mol for a singly charged ion of radius 1 Å. This large energy requirement is due to the occurrence of repulsive 'image charges' as the low dielectric medium is approached. Titrating charges on the melittin molecule should thus decrease the free energy expenditure for bringing the peptide to the interface, and thus increase the partition coefficient (by a factor of about 30 per charge titrated, according to the above estimate). This is in sharp contrast to the experimental observations. Our results therefore suggest a location of the melittin charges relatively far from the interface, largely exposed to water, 'seeing' a similar environment as on the surface of the aqueous peptide. Such a conclusion agrees with results obtained using spin-labelled melittin [22]. It is also in accord with recent NMR data showing only minor variation of lysine pK values between the aqueous and membrane-associated state of melittin [5]. Stronger changes in pK should be expected if the charges were to be introduced into an environment of low dielectric constant. Lysine and arginine side chains are clearly long enough to support a localization of their charged ends far from the interface, but still consistent with a deep insertion of the peptide backbone in the hydrophobic core of the bilayer.

Role of charged residues in the build-up of a surface potential

Melittin has long been known to interact much more strongly with negatively charged than with zwitterionic bilayers. Only recently, however, has the electrostatic contribution of the peptide–membrane interaction been explored in a quantitative way [6,8]. It turns out that the electrostatic component is equivalent (at neutral pH) to two effective charges per peptide molecule brought into the interface, if analyzed with a Gouy-Chapman type treatment [6–8]. This figure is small compared to the actual number of 5–6 unit charges carried by melittin

under those conditions. To some extent, this discrepancy can probably be explained by limitations of the model chosen, especially the assumption that charges are treated as being smeared homogeneously at the interface [23,24]. There must be more physical reasons involved, however. Recent NMR analysis has demonstrated that the effect of membrane-associated melittin on the head group conformation of the lipid is consistent with an effective interfacial charge of about 2 (see Ref. 8 and discussion of related results therein). Reduction of the effective charge by a factor of two has also been reported for membrane binding of oligolysines [25].

The most obvious physical explanation of observing an effective charge $\nu = 2$ would be to postulate that only two of the charged groups contact the membrane, the others remaining in the aqueous phase, fully screened by counterions. Our data do not favor such a hypothesis. In fact, Fig. 1 clearly shows partial contributions to the surface charge effect at least from the amino terminus and several (probably all) of the three lysines. The detailed argumentation is as follows:

Titration of the amino terminus could be related to the change in the isotherms between pH 6.8 and 7.8, by comparison with N-terminally formylated melittin. A pK value in this range agrees well with previous work [5]. The corresponding change in ν is 0.4. From pH 7.8 to fully acetylated melittin, ν decreases by about 1.1 (Fig. 2, Table I). Several residues must be involved in this change, because the relatively smooth variation over a broad range of pH (cf. Fig. 1) cannot be due to titration of one single protonated group. Again, this is consistent with titrations done with NMR methods [5]. The three lysine side chains in melittin are in fact expected to have different pK values, the ones of Lys-21 and Lys-23 being decreased due to charge clustering in the carboxy-terminal segment of the peptide. It is tempting to assign the change of 1.1 units in ν in roughly equal proportion to the three lysine side chains. In this case, each ϵ-amino group would contribute a fraction of about 0.35–0.4 to the total interfacial charge, similar to that of the terminal α-amino group. In contrast, the two arginines (which remain charged in the acetylated form) together contribute about 0.4 units, not much more than a single amino group alone. In fact, their contribution could well be even less than that: at such a low value of ν, bending of the isotherm occurs only at r values well above 0.1, where non-ideal effects on the partition equilibrium would be expected even without electrostatic contributions [12]. If this were the case also for acetylated melittin, only part of the activity coefficient would be due to charge accumulation at the interface, and the value of ν correspondingly smaller. On the other hand, ν could be slightly underestimated if some vesicles were disrupted at the high r values so that melittin had access to the inner compartment.

We conclude that the two arginines in any case contribute little to the total interfacial charge. The simplest interpretation is that they are tightly associated with counterions or else positioned farther away from the interface than the lysines. (The latter hypothesis would contrast with the situation encountered with detergent micelles. There, the various charged groups of melittin are found at approximately equal distance from the interface [26]).

Having shown that at least the lysines and amino terminus contribute to the observed interfacial charge, the question turns up again why the observed value of ν is so low. Localization of the charges at some distance from the interface (e.g. of the order of one Debye length, or 9.6 Å at 0.1 M univalent electrolyte) would reduce the effective potential by nearly one half with respect to the one produced by charges at the interface [27,28]. Such ideas would be consistent with the conclusions drawn from the constancy of the partition coefficient. In the Appendix 2, we show that the effective reduction of charge can at least partly be rationalized in such a way.

Repeating some of our experiments at different salt concentrations, the most conspicuous result was the marked decrease of ν at 1.01 M ionic strength. Increased screening by counter-ions is not sufficient to explain this effect. Fig. 3 compares the measured isotherm to the one calculated from the 0.11 M data, adjusting the salt concentration to 1.01 M in the Gouy-Chapman formula. Obviously the measured curve bends much less than expected theoretically.

Going from 0.11 to 1.01 M ionic strength, the Debye length decreases by a factor of about 3. The distance from the interface or the size of the peptide, when measured in units of the Debye length, then increase correspondingly. Of course, other phenomena may also be involved, e.g., increased counter-ion association with the peptide and possibly effects due to aggregation of the membrane-associated peptide [30].

Acknowledgments

We thank Drs. G. Beschiaschvili, E. Kuchinka and H. Vogel for discussions and for supplying samples of natural and modified melittin, and Ms. P. Savko for technical assistance. This work was supported by grant No. 3-285.85 of the Swiss National Science Foundation.

Appendix 1

Correction for tetramerization of melittin in aqueous solution

The ellipticity (per residue) of melittin in aqueous electrolyte solution is given by

$$\theta_w = (c_1\theta_1 + 4c_4\theta_4)/c_w \qquad \text{(A-1)}$$

with θ_1, θ_4 being the ellipticities of monomeric and tetrameric peptide, respectively. c_1 and c_4 are the corresponding concentrations, and $c_w = c_1 + 4c_4$ stands for the total aqueous concentration. The mass action law relates c_1 and c_2 via the tetramerization constant, K_a:

$$4K_a c_1^4 = (c_w - c_1) \tag{A-2}$$

Having determined θ_1 and θ_4, K_a can be evaluated from a measurement of θ_w as a function of c_w (see Ref. 11 for details). If vesicle material is added to the peptide solution, the observed ellipticity signal, θ, is given by

$$\theta = (\theta_w c_w + \theta_\infty c_a)/c_{tot} \tag{A-3}$$

(θ_∞ is the ellipticity of vesicle-associated peptide, $c_a = rc_L$ its concentration, and $c_{tot} = c_a + c_w$). Of course, c_w decreases upon association of some peptide material with the vesicles leading to a shift of the monomer-tetramer equilibrium in favor of the monomers. Under the conditions used in this study, tetramer formation was always negligible in the presence of higher amounts of lipid, so that at most the very first points in a titration had to be corrected. Thus, there were no problems in determining θ_∞. Correction for the first point in a titration was as follows: Let $\theta_w^{(0)}$ be the value of θ_w before addition of the vesicles, and θ the ellipticity measured after the addition. A first estimate of c_a/c_{tot} was then obtained from $(\theta - \theta_w^{(0)})/(\theta_\infty - \theta_w^{(0)})$, according to Eqn. A-3. From this resulted a value of $c_w = c_{tot} - c_a$, then c_1 by (numerically) solving Eqn. A-2 and finally a new value, $\theta_w^{(1)}$, for the ellipticity of the aqueous peptide, lower in magnitude than $\theta_w^{(0)}$. The procedure was repeated, using $\theta_w^{(1)}$ instead of $\theta_w^{(0)}$ to obtain a new value, $\theta_w^{(2)}$, and so on, until θ_w remained constant to within 1%, well below experimental error. For the second titration step, the θ_w of the previous step was used as the starting value. The procedure could be speeded up considerably by using better guesses of the initial values, guided by the results obtained in previous calculations.

Appendix 2

This appendix is intended to show how the distance of charged groups from the interface can lead to a reduction of physical charge ze_0 to an effective value νe_0. (Experimentally, $\nu = 0.4\ z$ for the amino groups, according to the results presented above; the even stronger reduction in the case of the arginines may then be rationalized as outlined in Discussion). Distance from the interface can be treated in an extended Gouy-Chapman calculation (see Refs. 24 and 29 for relevant references). We choose a particularly simple model which is fully sufficient to demonstrate the relevant aspects. Due to the basic assumptions inherent in a Gouy-Chapman type model, it is not possible within that framework to deal with finite peptide size. Work is in progress to include that aspect, too.

Extended Gouy-Chapman model

We assume the surface charge to be placed not at the interface of the membrane, but in a plane parallel to it, at a distance d. Ions are thought to penetrate through this plane up to the interface. We limit our considerations to small surface potentials so that the linearized form of the Poisson-Boltzmann equation can be applied. Calculation is then straightforward along the lines indicated, e.g., in Ref. 28. The potential is found to decay exponentially from the charged plane into the bulk solution, the decay length being equal to the Debye length, κ^{-1}, as in ordinary Gouy-Chapman theory. Between the membrane and the charged plane, the potential is given by the sum of two exponentials, $B\exp(-\kappa x) + C\exp(\kappa x)$. The constants are determined by the boundary conditions (continuity of ξ at the charged plane, its derivatives from the two sides combining to $-\sigma/\varepsilon\varepsilon_0$ whereas the derivative of ψ vanishes at the uncharged interface). At the charged plane we find the following value:

$$\psi = \psi_0(1 + \exp[-2\kappa d])/2 \tag{A-4}$$

where ψ_0 is the usual Gouy-Chapman potential obtained if the same surface charge density were assembled at the interface, $d = 0$. It is evident, that the effective potential is nearly halved with respect to ψ_0 if the charges are localized about one Debye length or more from the interface. Of course, this is due to screening by counter-ions from both sides of the charged plane, whereas screening is only possible from one side at the interface itself. Other charge distributions, e.g., a homogeneous space charge in a region of width d adjacent to the membrane, lead to numerically similar results [27,28].

This kind of model thus gives an effective charge reduction of at most a factor of two, even if the salt concentration is strongly increased. We are therefore working on alternative models in order to account for other factors, e.g., the size of the peptide to which the charges are attached.

References

1 Jähnig, F., Vogel, H. and Best, L. (1982) Biochemistry 21, 6790–6798.
2 Tosteson, M.T. and Tosteson, D.C. (1984) Biophys. J. 45, 112–114.
3 Morgan, C.G., Williamson, H., Fuller, S. and Hudson, B. (1983) Biochim. Biophys. Acta 732, 668–674.
4 Batenburg, A.M., Van Esch, J.H. and De Kruijff, B. (1988) Biochemistry 27, 2324–2331.
5 Stanislawski, B. and Rüterjans, H. (1987) Eur. Biophys. J. 15, 1–12.

6 Schwarz, G. and Beschiaschvili, G. (1989) Biochim. Biophys. Acta 979, 82–90.
7 Kuchinka, E. and Seelig, J. (1989) Biochemistry 28, 4216–4221.
8 Beschiaschvili, G. and Seelig, J. (1989) Biochemistry 29, 52–58.
9 Kempf, C., Klausner, R.D., Weinstein, J.N., Van Renswoude, J., Pincus, M. and Blumenthal, R. (1982) J. Biol. Chem. 257, 2469–2476.
10 Vogel, H. and Jähnig, F. (1986) Biophys. J. 50, 573–582.
11 Schwarz, G. and Beschiaschvili, G. (1988) Biochemistry 27, 7826–7831.
12 Rizzo, V., Stankowski, S. and Schwarz, G. (1987) Biochemistry 26, 2751–2759.
13 Dasseux, J.L., Faucon, J.F., Lafleur, M., Pézolet, M. and Dufourcq, J. (1984) Biochim. Biophys. Acta 775, 37–50.
14 Dufourcq, J., Faucon, J.F., Fourche, G., Dasseux, J.L., Le Maire, M. and Gulik-Krzywicki, T. (1986) Biochim. Biophys. Acta 859, 33–48.
15 McLaughlin, S.A. (1987) Curr. Top. Membr. Transp. 9, 71–144.
16 Vogel, H. (1981) FEBS Lett. 134, 37–42.
17 Terwilliger, T.C., Weissman, L. and Eisenberg, D. (1982) Biophys. J. 37, 353–361.
18 Dawson, C.R., Drake, A.F., Helliwell, J. and Hider, R.C.(1978) Biochim. Biophys. Acta 510, 75–86.
19 Strom, R., Podo, F., Crifo, C., Berthet, C., Zulauf, M. and Zaccai, G. (1983) Biopolymers 22, 391–397.
20 Vogel, H., Jähnig, F., Hoffmann, V. and Stümpel, J. (1983) Biochim. Biophys. Acta 733, 201–209.
21 Von Heijne, G. and Blomberg, C. (1979) Eur. J. Biochem. 97, 175–181.
22 Altenbach, D. and Hubbell, W.L. (1988) Proteins 3, 230–242.
23 Schoch, P. and Sargent, D.F. (1980) Biochim. Biophys. Acta 602, 234–247.
24 McLaughlin, S.A. (1989) Annu. Rev. Biophys. Chem. 18, 113–136; p. 125.
25 Chung, L., Kaloyanides, G., McDaniel, R., McLaughlin, A. and McLaughlin, S. (1985) Biochemistry 24, 442–452; p. 450.
26 Brown, L.R., Braun, W., Kumar, A. and Wüthrich, K. (1982) Biophys. J. 37, 319–328.
27 Ohshima, H. and Ohki, S. (1985) Biophys. J. 47, 673–678.
28 Levine, S., Levine, M., Sharp, K.A. and Brooks, D.E. (1983) Biophys. J. 42, 127–135.
29 Honig, B.H., Hubbell, W.L. and Flewelling, R.F. (1986) Annu. Rev. Biophys. Biophys. Chem. 15, 163–193.
30 Bradrick, T.D., Freire, E. and Georghiu, S. (1989) Biochim. Biophys. Acta 982, 94–102.