Biochimica et Biophysica Acta 861 (1986) 141–151
Elsevier

BBA 73286

Thermodynamic analysis of incorporation and aggregation in a membrane: Application to the pore-forming peptide alamethicin

Gerhard Schwarz, Stefan Stankowski and Vincenzo Rizzo

Department of Biophysical Chemistry, Biozentrum, University of Basel, Klingelbergstrasse 70, CH-4056 Basel (Switzerland)

(Received 12 March 1986)

Key words: Activity coefficient; Alamethicin; Partition coefficient; Peptide–lipid interaction; Thermodynamics; Membrane binding; Circular dichroism

Interaction of the pore-forming antibiotic alamethicin with small unilamellar vesicles of dioleoylphosphatidylcholine has been studied by means of circular dichroism. The data strongly suggest that alamethicin does not bind to the surface of the vesicles but incorporates into the lipid phase to a fairly large extent. Furthermore, aggregation of the peptide in the membrane is apparent from the existence of a 'critical concentration'. Quantitative evaluation and interpretation of the data rest on a quite generally applicable thermodynamic analysis. The underlying phenomenon is treated in terms of a partition equilibrium between the aqueous and lipid media. In the bilayer phase non-ideal interactions (described by appropriate activity coefficients) as well as aggregate formation are considered. Using this approach the relevant parameters of the alamethicin-lipid system have been determined (yielding, in particular, a partition coefficient of $1.3 \cdot 10^3$ for the monomeric peptide and a critical aqueou sconcentration of 2.5 μM). Finally, the possible relevance of these results for the voltage-dependent gating of alamethicin is briefly pointed out.

Introduction

There is a great variety of substances with an apparent affinity for phospholipid bilayers. The underlying interactions may be of biological significance. In the past years a wealth of data about the physical chemistry of pertinent systems has been accumulated (e.g. Refs. 1–11). Interpretation of these data has so far mainly been based on very simple thermodynamic approaches, particularly the application of elementary binding, adsorption or partioning models [1–6]. In many cases such simplification appears to be quite unsatisfactory out of mere a priori considerations.

First of all we shall clearly distinguish between binding and incorporation. The former mode is to include adsorption and implies the existence of saturable sites of interaction. On the other hand, incorporation must rather be regarded as a mixing process where the lipid plays the part of solvent.

In contrast to the fundamental idea of the respective elementary models, the individual substrate molecules frequently do not act independently on the membrane. In the case of binding this can be described by making allowance for cooperative effects [12]. If the substrate incorporates, aggregate formation may be a complicating factor. Under any circumstances, however, thermodynamically non-ideal behavior is an essential point of concern if the substrate becomes sufficiently concentrated within the lipid phase. The incorporated molecules then have comparatively close distances resulting in solute-solute interaction which has to be taken into account by ap-

Correspondence address: Department of Biophysical Chemistry, Biozentrum, University of Basel, Klingelbergstrasse 70, CH-4056 Basel, Switzerland.

propriate activity coefficients.

We became fully aware of the practical importance of these problems while we were studying the association of the pore-forming peptide alamethicin [13–15] with phospholipid vesicles. In order to understand the data, aggregation of incorporated peptide as well as non-ideal solution characteristics proved to be necessary components in a quantitative interpretation.

In this article we first report on some experimental data with dioleoylphosphatidylcholine and alamethicin. They are then evaluated using suitable methods which will be briefly explained and demonstrated. After having presented the final results and conclusions, more detailed theoretical argumentations are given to substantiate our approach. The latter is thought to be of quite general advantage for a wide range of applications when incorporation into lipid bilayers is being investigated.

Experimental

Materials and Methods

1,2-Dioleoyl-*sn*-glycero-3-phosphorylcholine (DOPC) was purchased from Avanti Polar Lipids (Birmingham, AL) and used without further purification. Tris was a Merck product. Quartz-distilled water was used for the preparation of solutions. Alamethicin was purified from raw extracts of *Trichoderma viride* culture medium (donated by Dr. G. Jung, Institute of Organic Chemistry, University of Tübingen, F.R.G.). More details of the purification (adapted from Ref. 16), and the characterization of alamethicin will be reported in a forthcoming article (V. Rizzo, S. Stankowski and G. Schwarz, unpublished data).

Alamethicin stock solutions in CH_3OH/H_2O (1 : 3) at a concentration of 1–1.5 mg/ml could be stored at 4°C for several weeks. The concentration of alamethicin solutions was determined from the circular dichroism (CD) spectrum on the basis of a mean residue ellipticity $[\theta]_{220} = -12750$ deg $\cdot$ dmol^{-1} $\cdot$ cm^2 in methanol at 20°C, and $[\theta]_{224} = -4750$ deg $\cdot$ dmol^{-1} $\cdot$ cm^2 in water at 20°C. Stock solutions of lipid vesicles were prepared the day of the experiment by sonicating a lipid dispersion (5 mM) under argon with a titanium tip sonifier for about 30 min. The temperature was kept around 25°C with a thermostatting jacket. The optically clear solutions so obtained were then centrifuged in an Eppendorf centrifuge for 10 min in order to remove metallic titanium. The concentration of DOPC was determined by phosphate analysis [17] with an accuracy of $\pm 5\%$.

CD measurements were carried out on an extensively modified version of a Cary 61 instrument [18], which was calibrated with a sample of D-(+)-10-camphorsulfonic acid of known chemical and optical purity. Titrations were performed by microliter additions of a concentrated vesicle solution to an alamethicin solution in a thermostatted rectangular fused-silica cuvette of 1 cm path length. Alamethicin was also present in the vesicle solution in order to maintain its concentration constant in the course of a titration experiment. The CD contribution of the lipid vesicles was determined in an independent experiment and subtracted from the titration data. Baseline shifts were checked by monitoring the CD signal at 260 nm, where alamethicin shows no significant contribution.

The total concentrations of lipid and peptide are denoted c_L and c_P, respectively. The amount of peptide associated with the lipid is expressed in terms of the ratio of the molar concentrations in question, $c_a/c_L = r$. The 'free' (aqueous) peptide concentration, c_f, accordingly can be determined as

$$c_f = c_P - r \cdot c_L \tag{1}$$

The circular dichroism signal of aqueous alamethicin was found to increase strictly linearly with c_f in the range of concentrations used (2–16 μM). Upon adding lipid vesicles, the trough of the alamethicin CD spectrum near 220 nm (which is typical of α-helical contributions) was strongly enhanced. We have chosen the increment of negative ellipticity per mean residue at 224 nm as our actual signal, F, to measure the peptide/lipid association. The data could be interpreted on the basis of a simple two-state model involving a characteristic value F_∞ for the case that all the peptide is associated (whereas $F = 0$ if all the peptide is free). Thus we have generally $F = F_\infty(rc_L/c_P)$ so that

$$r = (F/F_\infty) \cdot (c_P/c_L) \tag{2}$$

can be employed to calculate r from the experimental data (provided F_∞ is known).

Results

Fig 1 presents our measured signal, F, versus the total lipid-to-alamethicin ratio c_L/c_P for various values of c_P. Most striking is the fact that the individual curves level off towards markedly reduced plateau values when c_P is decreased. The apparent plateaus ($F \to F'_\infty < F_\infty$) reflect some peptide being retained in the aqueous phase even if the lipid phase is further extended. In other words, the aqueous peptide concentration at $c_L \to \infty$ appears to approach a finite value, i.e., $c_f \to c_f^0 > 0$. Such a 'critical concentration' effect strongly suggests the existence of an aggregation process. This will be confirmed by a quantitative treatment to be described presently.

To begin with, we need to know F_∞, which can in principle be determined by measuring F at sufficiently large c_L. A straightforward extrapolation is, however, not feasible in our case since the lipid concentrations used in the measurements are restricted because of the need to keep down light-scattering artefacts. The plateaus turn out to increase with c_P even at the highest experimentally possible c_L, suggesting that they still lie appreciably below the final F_∞.

Nevertheless we have devised a practicable procedure to find out F_∞. It can be shown from thermodynamic principles that a definite functional relationship

$$r = \phi(c_f) \quad (\text{with } r \to 0 \text{ at } c_f \to 0) \tag{3}$$

exists, i.e. the associated peptide-to-lipid ratio is directly controlled by the free peptide concentration (see Theoretical section below). The qualitative course of a pertinent r vs. c_f isotherm is shown in Fig. 2. Mass conservation according to Eqn. 1, on the other hand, corresponds to a straight line in the same diagram. It intersects the thermodynamic association isotherm at the actual values of r and c_f which the system assumes for given total concentrations of peptide and lipid. Then, upon raising c_L in a range of moderate r, extrapolation of c_f along a linear approximation of the ϕ function will result in $rc_L \to c_P - c_f^0$. In terms of the original signal this is equivalent to

$$F \to F'_\infty = F_\infty \cdot \left(1 - \frac{c_f^0}{c_P}\right) \tag{4}$$

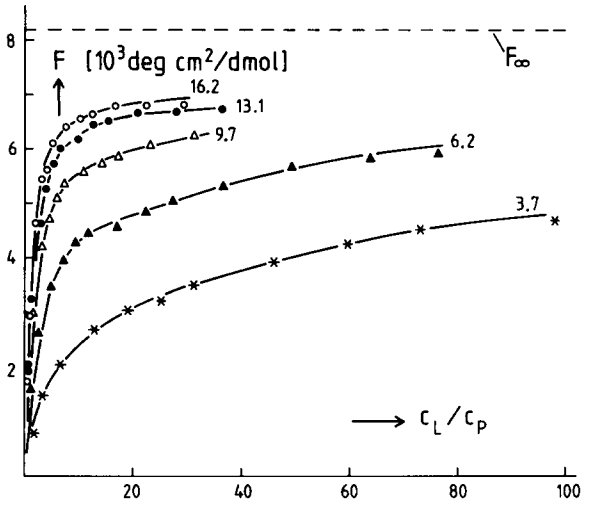

Fig. 1. Enhancement of the mean residual negative ellipticity at 224 nm, F, of alamethicin upon addition of DOPC vesicles (c_L/c_P is the total lipid-to-peptide ratio). Conditions: 21°C, 10 mM Tris-HCl buffer of pH 7.4, total alamethicin concentrations (in μM) as indicated for each curve. Solid curves are drawn solely to guide the eye. The upper bound F_∞ (dashed line) refers to the case where peptide is fully associated with the lipid (see text and Fig. 3).

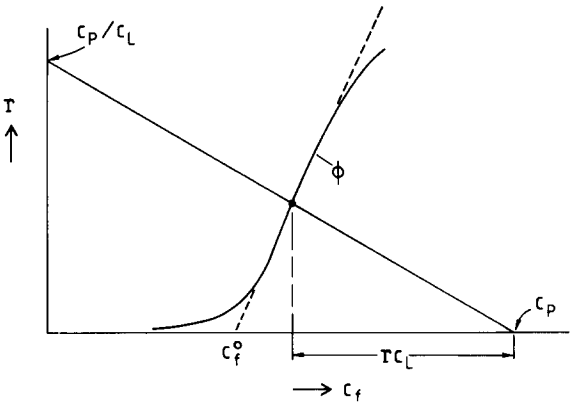

Fig. 2. Graphical method to determine the free and associated peptide concentrations (see text). The sigmoidal shape of the appropriate thermodynamic association function ϕ (solid curve) is typical for either a cooperative binding effect or an incorporation in the lipid phase coupled with internal aggregation.

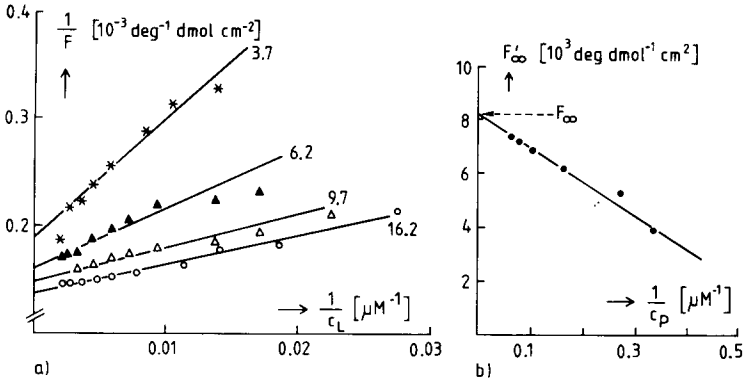

Fig. 3. Procedures to determine F_∞ (see text): (a) double reciprocal plots of data from Fig. 1 with straight lines intercepting the ordinate axis at $1/F'_\infty$; (b) the resulting $F_\infty = 8200$ deg·cm²·dmol^{-1} (data points are included which are omitted in other figures for the sake of clarity).

The above linear approximation (dashed line in Fig. 2) is described by $r = k_0 \cdot (c_f - c_f^0)$ with an appropriate slope k_0. Considering the Eqns. 1 and 2 this eventually yields

$$\frac{1}{F} = \frac{1}{F'_\infty} \cdot \left(1 + \frac{1}{k_0 c_L}\right)$$

Thus we conclude that F'_∞ can be determined by a double reciprocal plot of F versus c_L. Actually such a procedure is perfectly analogous to well-known standard methods [19], apart from the fact that the extrapolation involves a non-zero value of c_f^0. The situation is fully comparable to the case of micelle formation and similar aggregation phenomena [20]. Double reciprocal plots of the kind proposed are shown in Fig. 3a. They are indeed linear as predicted, except for distinct deviations from a straight line at fairly small $1/c_L$ as observed for the lowest c_P. This reflects the fact that the range of $c_f \lesssim c_f^0$ is reached where the ϕ isotherm bends so that the chosen linear approximation of ϕ no longer applies (see Fig. 2). In view of Eqn. 4, the F'_∞ values obtained from these plots can be further evaluated by means of another straight line when plotted versus $1/c_P$. The intercept on the ordinate then yields $F_\infty = 8.2 \cdot 10^3$ deg · dmol^{-1} · cm² (see Fig. 3b).

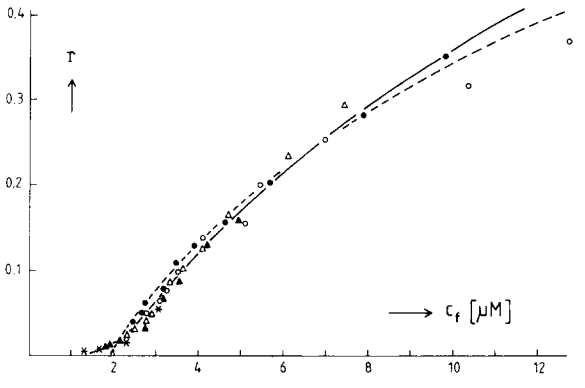

Fig. 4. The data of Fig. 1 have been plotted as the molar ratio of associated peptide to lipid, r, versus the free aqueous peptide concentration, c_f. The curves are drawn according to our non-ideal approach describing incorporation coupled with internal aggregation (see text). Even the simple limiting function of Eqn. 9 formally provides a rather good fit (dashed curve; $c_f^* = 1.9$ μM, $z = 3.2$). In order to fit the bend of the curve at low r also, we have assumed a minimum aggregate size of $m = 5$ and then calculated the solid curve with $c_f^* = 2.5$ μM, $K = 500$ (implying $\Gamma_1 = 1.0 \cdot 10^3$ M^{-1}) and $z = 2.7$ (z_1 was the same as z, but can actually be varied over a broad range without significant effect on the curve). Satisfactory fits are also possible for other m values (and somewhat modified K, Γ_1).

Using this value of F_∞ we have obtained r vs. c_f isotherms at 21°C as presented in Fig. 4. The points clearly coincide for all c_P studied, in accordance with Eqn. 3, confirming the consistency of our approach. Despite the high values of $r > 0.3$ reached at large c_f, the curves show no indication of saturation. Similar experiments have been carried out at 11°C and 36°C. No significant effect of temperature could be observed.

Discussion

First we examine whether the observed r vs. c_f curve possibly reflects binding to the lipid bilayer. Because of the sigmoidal course this would then imply cooperative effects. Anyway, the data in Fig. 4 call for a saturation limit of r clearly above 0.3. This corresponds to much less than two lipid headgroups per peptide molecule in either one of

the two leaflets. One such headgroup comprises an area A_L of approximately 0.70 nm^2 [21]. Thus a binding site could hardly cover more than 1 nm^2. On the other hand, the peptide molecule can be assumed to have an elongated shape with a diameter of at least 1 nm and length of about 3.5 nm [22]. Accordingly binding parallel to the lipid/water interface, which would require a minimum area of 3.5 nm^2, can be definitely ruled out. However, the cross-sectional area A_P of around 0.75 nm^2 basically allows a binding mode perpendicular to the membrane but then the peptide molecules must become extremely crowded under saturation conditions. Instead of this very unlikely possibility we believe that the peptide actually penetrates the hydrophobic core of the bilayer. This is consistent with the observed change of the circular dichroism which can be interpreted most naturally as resulting from the enhanced secondary structure of alamethicin upon transfer from the aqueous to the lipid environment. Similar effects have indeed been found in solvents of reduced polarity [23]. Incorporation of the peptide is also indicated by the established pore formation and its strong increase with the aqueous alamethicin concentration [14]. Furthermore, it has been demonstrated by means of cross-linkers [24] that the peptide indeed penetrates deeply into the membrane even in the absence of an electrical potential.

We therefore treat our data in terms of a partition equilibrium of the peptide (as a solute) between the water and lipid phases. Aggregation of the peptide within the lipid moiety must be postulated in order to explain the initial lag and the subsequent bending upwards exhibited by the r vs. c_f isotherm. This shape is certainly not due to aggregation in the aqueous medium, which would definitely tend to cause a quite different trend (no sigmoidal course and bending in the opposite direction). Moreover, we could not find any clue for aggregate formation of the free peptide as judged from the above mentioned linear c_f-dependence of the circular dichroism signal. Accordingly the peptide is assumed to be practically monomeric in its aqueous state.

For a quantitative interpretation we need a suitable thermodynamic formalism. The detailed approach will be developed below in the Theoretical section. Basically it considers non-ideal effects as well as a simplified aggregation model for the peptide in the membrane. The possible aggregation numbers range from a minimum of m to infinity. The equilibrium constant of each aggregation step is taken to have the same value K (isodesmic model). Actually a fitting of the data is not very sensitive to the particular model used (isodesmic, micellar or mixed forms). Our present choice is motivated by the established occurrence of a distribution of large pore sizes [25] and the wish to limit the number of free parameters. To make allowance for non-ideal solute-solute interaction activity coefficients α_i are introduced (i being the aggregation number; $i = 1$ refers to the monomer). Thermodynamic activities $\alpha_i r_i$ (where r_i is the individual aggregate to lipid ratio) are then employed in the quantitative treatment of the partition and aggregation equilibria. In the absence of an explicit temperature effect we have simply

$$\alpha_i = (1+2r)^{z_i} \tag{5}$$

where z_i stands for a specific number (resulting from the underlying statistical thermodynamical argumentation given in the Theoretical section). Along these lines the amount of incorporated monomers is described by

$$r_1 = (\Gamma_1/\alpha_1)\cdot c_f \tag{6}$$

where Γ_1 denotes the proper (ideal) partition coefficient. In the context of our aggregation model the z_i parameter is assumed to change by a constant increment z per aggregation step. Then one eventually obtains

$$r_i = s^{i-1}\cdot r_1 \quad \text{where } s = (c_f/c_f^*)/(1+2r)^z \tag{7a,b}$$

involving a critical concentration

$$c_f^* = 1/(\Gamma_1 K) \tag{8}$$

We note that always $s < 1$. Without an activity correction term the r vs. c_f isotherm therefore cannot continue beyond c_f^* (see Fig. 6), but bends upwards more and more when that boundary is approached. Such a course can never fit our data of Fig. 4. This emphasizes the importance of taking into account a non-ideal behavior of the system.

At sufficiently large r where virtually all incorporated peptide is aggregated we have practically $s = 1$. Accordingly Eqn. 7b suggests the existence of a limiting isotherm

$$c_f = (1+2r)^z \cdot c_f^* \qquad (9)$$

In fact our data can thus be fitted very well (dashed curve) with an adjusted $c_f^* = 1.9$ μM, $z = 3.2$ which are determined from a linear plot of $\ln c_f$ vs. $\ln(1 + 2r)$. More rigorously, however, we must consider that s lies slightly below unity, particularly at smaller r where an appreciable fraction of monomeric peptide occurs. This implies that actually c_f^* must be somewhat increased while z is decresed. From the measured r at $c_f \lesssim c_f^*$ a partition coefficient $\Gamma_1 \approx 1.0 \cdot 10^3$ M^{-1} can be estimated assuming a value of $m = 5$. A very satisfactory fit is then possible in the full range of measured points (see solid curve in Fig. 4). The appropriate parameters are $c_f^* = 2.5$ μM, $K = 500$ and $z = 2.7$ (the value of z_1 proves to be insignificant).

Note that our partition coefficient Γ_1 has been defined using the peptide-to-lipid molar ratio as the concentration variable in the membrane. Switching to mol per liter yields an ordinary partition coefficient $\gamma_1 = \Gamma_1/\bar{V}_L \approx 1.3 \cdot 10^3$ ($\bar{V}_L = 0.79$ dm^3/mol is taken as the partial molar volume of the DOPC in vesicles according to data reported for similar systems [26]). Accordingly the standard free energy change upon a transfer of the peptide from the aqueous to the lipid bilayer phase is -17.5 kJ/mol. Since we could not detect a significant temperature effect, the affinity of alamethicin for the membrane should be due to an increase of entropy. This appears to be caused by (de)solvation processes creating disorder which greatly overcompensates the effect of entropy decrease associated with the enhanced secondary structure of the peptide.

Anyway, having obtained an approximate value of Γ_1, we can fairly well estimate the amounts of monomer and aggregates in the bilayer at any given external concentration c_f. Let us choose for example 0.1 μM (which is of the order of magnitude employed in electrical pore-forming experiments [13,14]). This means there are $6 \cdot 10^{13}$ peptide molecules per ml in the aqueous phase which is supposed to be in contact with a small DOPC black membrane. A comparatively very small number of the molecules, namely $2.9 \cdot 10^8$ per mm^2, will enter the bilayer (1 peptide per 10000 lipids). These are practically all monomeric. The number of aggregates per mm^2 turns out to be for example 730 ($i = 5$) and 0.05 ($i = 8$). Nevertheless these numbers are drastically increased by even a small membrane potential if the peptide is incorporated in the membrane with its permanent electric dipole moment $\boldsymbol{\mu}_1$ parallel to the membrane field as a result of the electrostatic energy contribution $(-\boldsymbol{\mu}_1 \cdot \boldsymbol{E})$ to the total free energy of the peptide in its incorporated state. Therefore, at a field strength $\boldsymbol{E}$, the partition coefficient will be enhanced by a Boltzmann factor of $\exp(\boldsymbol{\mu}_1 \boldsymbol{E}/kT)$ whereas the critical concentration decreases by the same factor (provided there is no change of dipole moment upon aggregation). Suppose we have an applied membrane potential of 30 mV (so that $\boldsymbol{E} = 100$ kV/cm assuming a distance of about 3 nm across the apolar core of the membrane [21]) and an alamethicin dipole of 80 Debye units (approximately as measured in octanol [22]). Under such conditions the zero-field value of Γ_1 is doubled (and the critical concentration drops to one-half of its original value). The numbers of each aggregate will increase by a factor of 2^i. Thus the equilibrium number of aggregates composed, for instance, of 8 single peptides should rise from 0.05 per mm^2 to about 6. It may therefore be possible that the voltage-dependence of the alamethicin gating phenomenon primarily reflects an electric field effect on the partition coefficient of the peptide.

Theoretical

Fundamental aspects

We consider n_L mol of lipid to which $n_a = r \cdot n_L$ mol of a certain substrate is somehow associated (at given temperature and pressure). The Gibbs free energy of the system, G, must be proportional to the total amount of material, provided the mixing ratio r is held fixed. In other words, we have generally $G = n_a \cdot g(r)$. Accordingly the chemical potential of the substrate under any circumstances becomes a definite function of the variable r: $\mu_a = g + r \cdot (\partial g/\partial r)$, as obtained by

partial differentiation of G with respect to n_a at constant n_L. For sufficiently high dilution (i.e. $r \to 0$) one finds

$$\mu_a = \mu_a^{\ominus} + RT \cdot \ln r \tag{10}$$

with an appropriate standard potential $\mu_a^{\ominus}$ which does not depend on r (see Eqn. 17 below). Deviations from this limiting function will occur at larger r according to the special nature of the association mechanism. These deviations are to be expressed in terms of a specific function $q(r)$ so that always

$$\mu_a = \mu_a^{\ominus} + RT \cdot \ln(q \cdot r)$$

Let us now consider possible substrate exchange with an aqueous medium where the concentration, c_f, remains small enough to allow disregard of non-ideal solute-solute interactions. In that medium the chemical potential of the substrate may thus be written

$$\mu_f = \mu_f^{\ominus} + RT \cdot \ln c_f$$

When equilibrium is established we have $\mu_f = \mu_a$ and readily obtain

$$r \cdot q(r) = \Gamma c_f \quad \text{where} \quad \Gamma = \exp\left\{\left(\mu_f^{\ominus} - \mu_a^{\ominus}\right)/RT\right\} \tag{11a,b}$$

The quantity Γ stands for a generally defined 'partition coefficient'. This formulation of c_f as a function of r can be inverted to yield a pertinent thermodynamic association isotherm

$$r = \phi(c_f) \tag{12}$$

We therefore conclude that irrespective of the special mechanism of substrate-lipid association a definite functional relationship must exist between the associated substrate-to-lipid ratio and the 'free' aqueous concentration.

First we shall envisage the case of negligible non-ideal interactions between the associated substrate molecules. If the substrate incorporates in the bilayer, a pure mixing mode of association prevails. Then we have $q = 1$ and accordingly $r = \Gamma c_f$. In other words, a simple 'linear' partition equilibrium is encountered. A binding mode, however, will eventually exhibit a different course of r versus c_f. Let us assume r_b equivalent and independent binding sites per lipid and a relevant binding constant K_b so that $r = K_b \cdot c_f \cdot (r_b - r)$ according to the ideal mass action law. This leads to $r/r_b = K_b c_f/(1 + K_b c_f)$ which conforms to Eqn. 15 if we set

$$q(r) = r_b/(r_b - r), \quad \Gamma = K_b r_b \tag{13}$$

A linear course of r vs. c_f apparently applies here if $r \ll r_b$ (i.e. $K_b c_f \ll 1$). With increasing r the curve levels off and approaches r_b asymptotically (see Fig. 5). More complicated binding modes can be treated analogously.

Leaving behind the possibility of binding we note that an apparent $q(r)$ may also be caused by diverse types of substrate-substrate interactions. Among them are aggregation processes. As an example we shall briefly examine a dimerization of the incorporated substrate. Provided further non-ideal substrate-substrate interactions are negligible, we have according to ideal mass action a dimer-to-lipid ratio of $r_2 = K_d \cdot r_1^2$ (where K_d is the thermodynamic dimerization constant). The monomer-to-lipid ratio $r_1 = \Gamma_1 c_f$ is controlled by the aqueous substrate concentration via a linear

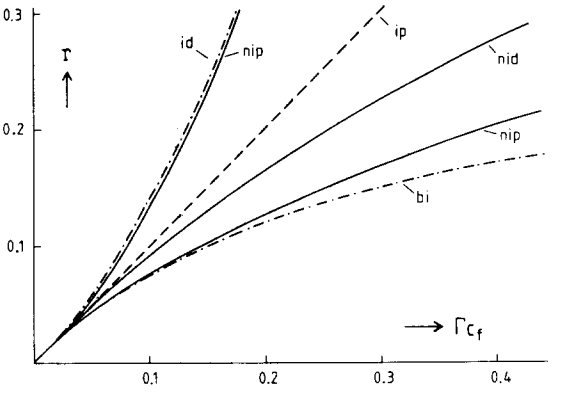

Fig. 5. Typical thermodynamic association functions according to Eqn. 11 for various elementary cases: ideal partitioning (ip), non-cooperative binding (bi) with $r_b = 0.3$ (see Eqn. 13), ideal dimerization (id) with $K_d = 2$ (see Eqn. 14). The effect of activity coefficients (Eqn. 21) is also indicated: (a) non-ideal partitioning curves of monomers according to Eqn. 22 (nip) bend to the right if $z_1 > 0$ or $h_1 < 0$, but to the left if $h_1 > 0$ (assuming $b = 2$ the two specimen curves have been calculated with $z_1 = 2$, $h_1 = 2$); (b) the effect of purely entropic interactions (i.e. $h = 0$) on the ideal dimerization curve is demonstrated by an example (nid) where $z_1 = 2$, $z_2 = 4$ (see Eqn. 24).

partition equilibrium (assuming practically no dimerization in the aqueous medium). The total substrate-to-lipid ratio then becomes

$$r = r_1 + 2r_2 = (1 + 2K_d \cdot \Gamma_1 c_f) \cdot \Gamma_1 c_f \qquad (14a)$$

which in terms of Eqn. 15 is expressed as

$$q(r) = 2/\left(1 + \sqrt{1 + 8K_d r}\right) \qquad (14b)$$

This implies a linear relationship $r \approx \Gamma_1 c_f$ below $K_d r \approx 0.01$. Above that range the r vs. c_f curve exhibits a gradually increasing slope contrary to the binding case (see Fig. 5).

Binding and aggregation are by no means the only significant aspects in the present context. We must not ignore the possible existence of all kinds of repulsive and/or attractive forces which are exerted once the substrate molecules get too close to each other. These are usually taken into account thermodynamically by introducing appropriate activity coefficients. Such quantities may play a decisive role in the analysis of incorporation and aggregation processes which occur in a lipid bilayer.

Chemical potentials

The molar free enthalpies of the pure lipid and substrate are denoted μ_L and μ_S respectively. Upon mixing the amounts n_L and n_a to form some kind of associated state, the system attains a free enthalpy

$$G = n_L \cdot \mu_L + n_a \cdot \mu_S + \Delta H - T\Delta S \qquad (15)$$

The changes of enthalpy, ΔH, and of entropy, ΔS, are to be calculated separately.

First we treat the case that the lipid very much exceeds the substrate. Then we may simply write $\Delta H = n_a \cdot \Delta H^\infty$ where ΔH^∞ is the enthalpy change in 'mixing' 1 mol of the substrate with an infinite amount of the lipid. Regarding the change of entropy we begin with the transfer of only one substrate molecule into the pure lipid medium. According to Boltzmann's equation this is associated with a ΔS of $\boldsymbol{k} \cdot \ln \Omega_0$, the quantity Ω_0 representing the factor by which the degree of degeneracy is altered in the process. The degree of degeneracy (called 'probability' in many textbooks) is actually the number of possible quantum states at the given energy level. In the present case (for one substrate molecule which can associate anywhere to the bilayer) this number must accordingly be expected to increase in proportion to the extent of the bilayer. In other words, we may set

$$\Omega_0 = N_L \omega \qquad (16)$$

with N_L being the total number of lipid molecules and a quantity ω which is independent of N_L. We note that ω is related to the standard entropy of mixing as will be apparent immediately. Provided N_a, the number of all the eventually associated substrate molecules, remains small enough so that they do not interfere with each other, the factor Ω_0 practically applies to the transfer of any of these molecules independently. Then we obtain altogether

$$\Delta S = \boldsymbol{k} \cdot \ln\left(\Omega_0^{N_a}/N_a!\right) = n_a \cdot R \cdot (1 + \ln \omega) - n_a \cdot R \cdot \ln r$$

(division by $N_a!$ is included to take into account the fact that the molecules are indistinguishable). Having inserted the present ΔH and ΔS in Eqn. 19 partial differentiation with respect to n_a yields

$$\mu_a = \mu_a^\ominus + RT \cdot \ln r, \quad \text{where } \mu_a^\ominus = \mu_S + \Delta H^\infty - RT \cdot \ln \omega \qquad (17a,b)$$

This formulation of the chemical potential of the associated substrate evidently holds in the limit of $r \to 0$ irrespective of the actual mode of association.

In the following we shall focus on the incorporation mode, where the lipid bilayer phase is formally considered as a two-dimensional solution system. This especially envisages the case of molecules spanning the bilayer, such as for instance certain proteins and peptides in a membrane. The 'infinite dilution' state of such a solute molecule is seen to occur in the case of perfect solvation, which implies that solely lipid can be found in a critical neighborhood domain. This solvation shell will necessarily be perturbed by the invasion of other solute molecules once their overall concentration is sufficiently increased. Owing to the resulting solute-solute interactions the ideal chemical potential of Eqn. 17 is no longer applicable. It

must be adjusted by introducing a specific activity coefficient.

In order to derive a practically useful functional expression for such quantities a simple heuristic approach will be followed. We consider j incorporated molecules and a total of N_L lipid molecules altogether in the two leaflets of the bilayer. The probability of finding a lipid molecule at a randomly chosen position in a given leaflet then becomes

$$w_j = \frac{\beta N_L A_L}{\beta N_L A_L + j A_S} = \frac{1}{1 + b(j/N_L)}, \quad b = \beta^{-1}(A_S/A_L) \tag{18}$$

(β, fraction of lipid in that leaflet; A_L, A_S, area per molecule covered by lipid and substrate, respectively).

Next we propose that the actual ΔH decreases in proportion to the overall lipid content. Thus

$$\Delta H = n_a \cdot \Delta H^\infty / (1 + b \cdot r)$$

for a finite solute-to-lipid ratio r ($= n_a/n_L$). Regarding the entropy change a modified Boltzmann term Ω_j (instead of the previous Ω_0) is taken into account when a substrate molecule is transferred into a lipid system where j other such molecules are already dissolved. We imagine that the lipid in the solvation shells does not fully contribute to Ω_j, in other words we set

$$\Omega_j = N_L^{(j)} \omega \tag{19}$$

with a reduced number $N_L^{(j)}$ against the total N_L in Eqn. 16. In our model a lipid molecule is counted among the $N_L^{(j)}$ if it keeps sufficient distance from the surrounding substrate. This means that a certain neighborhood must be occupied by lipid only. If that range can accommodate z lipid molecules, the probability of finding such a pure lipid neighborhood is w_j^z. Accordingly we describe the proper value of $N_L^{(j)}$ as $N_L \cdot w_j^z$. Incorporation of N_a substrate molecules one after another then results in a final

$$\Delta S = k \cdot \ln\left(\Omega_0^{N_a}/N_a!\right) - kz \cdot \sum_{j}^{1, N_a - 1} \ln\left(1 + b \cdot \frac{j}{N_L}\right)$$

With the present modified ΔH and ΔS we obtain a chemical potential of the incorporated substrate

$$\mu_a = \mu_a^\ominus + RT \cdot \ln(\alpha \cdot r) \tag{20}$$

(the standard potential $\mu_a^\ominus$ being the same as given in Eqn. 17b) involving an activity coefficient

$$\alpha = (1 + br)^z \cdot \exp\left(-\left[1 - \frac{1}{(1 + br)^2}\right] \cdot h\right) \tag{21}$$

where $h = \Delta H^\infty / RT$. In case the substrate forms aggregates composed of i monomers we must of course assign individual α_i to each kind of aggregate. These α_i will be characterized by appropriate parameters z_i and h_i. The value of b is not affected and r refers to the total incorporated substrate (assuming that aggregate formation does not appreciably change the area covered in the bilayer).

According to the definition in Eqn. 18 there is an ideal value of $b = 2$ (corresponding to an equal distribution of lipid among the two leaflets, i.e. $\beta = 0.5$, and equal areas of substrate and lipid). Naturally, the actual b could deviate somewhat from 2. These deviations, however, are practically compensated by adequate changes of z_i and h_i as long as only a limited range of r is concerned. This situation applies rather well to our above experimental case and presumably to other similar ones. For the sake of simplicity we thus normalize the z and h parameters in the way that they are related to $b = 2$. Since the h_i values depend explicitly on T, they may be neglected anyhow for the present system (which has no temperature effect). Therefore we set here $\alpha_i = (1 + 2r)^{z_i}$.

Partition equilibrium and aggregation

Let us return to the question of a lipid bilayer which can exchange substrate with an aqueous solution. For a simple incorporation mode of monomers involving no aggregation we can set $q = \alpha_1$ in Eqn. 7. Then we have

$$r = (\Gamma_1/\alpha_1)\, c_f \tag{22}$$

This alone implies substantial deviations from the ideal linear relationship between r and c_f once the substrate-to-lipid ratio is sufficiently increased to give rise to non-ideal solute-solute interaction. The

apparent partition coefficient, $\Gamma_{app} = r/c_f$, gradually decreases for any finite z_1 and/or negative h_1, whereas a positive h_1 tends to raise Γ_{app} at increasing c_f (see Fig. 5). Note that practically the same course of r versus c_f is found with a choice of either z_1 or a certain $h_1 < 0$. Therefore it will not be possible to determine definitely these parameters from a relevant experimental curve. Nevertheless their respective contributions should be separable by means of measurements at different temperatures because the h parameters depends explicitly on T, in contrast to the z parameter.

Also in Fig. 5 the present effect of an activity coefficient on the partition equilibrium of an incorporated monomeric substrate is compared with a basic binding mode according to Eqn. 13. In a limited range of concentration the curves resulting in either case may have a rather similar appearance. They must, however, eventually diverge. When a binding effect applies the value of r asymptotically approaches an upper boundary (reflecting the saturation of available binding sites). No such boundary exists if the substrate actually mixes with the lipid.

On the other hand, positive values of h_1 would result in a curve which largely resembles one that is obtained for an assumed dimerization as described by Eqn. 14 (see Fig. 5). However, this latter equation has yet to be corrected, since so far we have not made allowance for the activity coefficients of the involved components. Let us consider the formation of an aggregate from i monomers. The appropriate version of the mass action law will read

$$\alpha_i r_i/(\alpha_1 r_1)^i = K_i = \exp\left(-\left[\mu_i^{\ominus} - i\mu_1^{\ominus}\right]/RT\right) \qquad (23)$$

(for $i = 2, 3, \ldots$). With regard to a dimerization ($K_2 = K_d$) the previous 'ideal' version of $q(r)$ in Eqn. 14b accordingly changes to

$$q(r) = 2\alpha_1/\left\{1 + \sqrt{1 + 8K_d\left(\alpha_1^2/\alpha_2\right)\cdot r}\right\} \qquad (24)$$

It is demonstrated in Fig. 5 that this can easily lead to a course of r versus c_f falling substantially below the ideal straight line and displaying a more and more decreasing slope.

In view of our alamethicin system a series of aggregates larger than dimers must be brought into play. We propose a minimum aggregate size of $i = m$ ($\geqslant 2$) and a constant increment of the standard free enthalpy per aggregated monomer: $\mu_i^{\ominus} = \mu_1^{\ominus} + (i-1)\mu_*^{\ominus}$. This apparently implies an aggregation constant $K = \exp\{(\mu_1^{\ominus} - \mu_*^{\ominus})/RT\}$ so that

$$K_i = K^{i-1} \text{ for } i \geqslant m, \quad K_i = 0 \text{ for } i < m \qquad (25)$$

The exponential parameters associated with our α_i are imagined to depend on i in a way which can (at least approximately) also be described as a constant increment change, namely $z_i = z_1 + (i-1)\cdot z$, $h_i = h_1 + (i-1)\cdot h$. Then we have simply

$$\alpha_i = \alpha^{i-1}\cdot\alpha_1$$

where α is given by Eqn. 21 with the present step values z and h. After having inserted these K_i and α_i in Eqn. 23 we readily arrive at

$$r_i = s^{i-1}\cdot r_1 \quad \text{where } s = K\cdot(\alpha_1/\alpha)\cdot r_1 \qquad (26)$$

The monomers are subject to a partition equilibrium between the two solvent phases implying $\alpha_1 r_1 = \Gamma_1 c_f$ so that one may alternatively write

$$s = (c_f/c_f^*)/\alpha, \quad c_f^* = 1/(\Gamma_1 K) = \exp\left\{\left(\mu_*^{\ominus} - \mu_f^{\ominus}\right)/RT\right\} \qquad (27a,b)$$

As mentioned before we use the simplified formulation $\alpha = (1+2r)^z$ as far as our special alamethicin/DOPC system is concerned.

Employing Eqn. 26 the total substrate-to-lipid ratio can be calculated, eventually resulting in

$$r = r_1 + m\cdot r_m + (m+1)r_{m+1} + \ldots = r_1\cdot f_m(s)$$

where

$$f_m(s) = 1 + \left\{[m(1-s)+s]\cdot s^{m-1}/(1-s)^2\right\}$$

Since $r_1 = (\Gamma_1/\alpha_1)c_f = (\alpha/\alpha_1)\cdot s/K$ we are led to

$$K\cdot(\alpha_1/\alpha)\cdot r = s\cdot f_m(s) \qquad (28)$$

This relationship is most suitable to calculate theoretical r vs. c_f isotherms provided the parame-

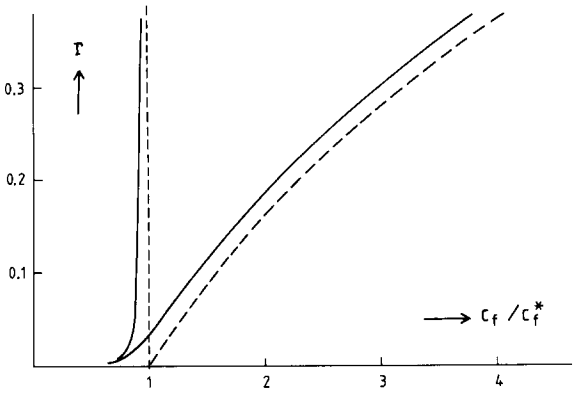

Fig. 6. Typical courses of r versus c_f according to our aggregation model (see text). In case non-ideal effects can be neglected, an extremely steep increase just to the left of the limiting line (dashed) at $c_f = c_f^*$ is indicated ($m = 5$, $K = 500$ for the solid curve). Solute-solute interactions which are purely entropic (i.e. $h = 0$, see Eqn. 21) imply a modified limiting curve (Eqn. 9) which definitely bends to the right (dashed curve, refers to $z = 2.5$). All curves for finite K (and Γ_1, respectively) take courses above it (the given solid curve has been calculated by means of Eqn. 28 with $m = 5$, $K = 500$; z_1 was 2.5 but is practically of minor significance).

ters of the system are given. Then we determine first the proper value of s for an individual value of r and subsequently compute $c_f = s \cdot \alpha \cdot c_f^*$ (according to Eqn. 27a). A graphical illustration of the present aggregation model is shown in Fig. 6.

Acknowledgments

This work was supported by grant No. 3.490.83 from the Swiss National Science Foundation. We greatly appreciate the help of Dr. G. Jung and Ms. U. Becker (University of Tübingen) at the preparation of alamethicin.

References

1 Kauffman, R.F., Chapman, C.J. and Pfeiffer, D.R. (1983) Biochemistry 22, 3985–3992
2 Vogel, H. (1981) FEBS Lett. 134, 37–42
3 Blatt, E. and Sawyer, W.H. (1985) Biochim. Biophys. Acta 822, 43–62
4 Lakowicz, J.R., Hogen, D. and Omann, G. (1977) Biochim. Biophys. Acta 471, 401–411
5 Burke, T.G. and Tritton, T.R. (1985) Biochemistry 24, 1768–1776
6 Witzke, N.M. and Bittmann, D. (1984) Biochemistry 23, 1668–1674
7 Davis, J.H., Clare, D.M., Hodges, R.S. and Bloom, M. (1983) Biochemistry 22, 5298–5305
8 El Karadaghi, S. et al. (1984) Biochim. Biophys. Acta 778, 269–275
9 Ikeda, T., Tazuke, S. and Watanabe, M. (1983) Biochim. Biophys. Acta 735, 380–386
10 Hartmann, W., Galla, H.J. and Sackmann, E. (1978) Biochim. Biophys. Acta 510, 124–139
11 Ernandes, J.E., Epand, R.M. and Schreier, S. (1983) Biochim. Biophys. Acta 733, 75–86
12 Stankowski, S. (1984) Biochim. Biophys. Acta 777, 167–182
13 Eisenberg, M., Hall, J.E. and Mead, C.A. (1973) J. Membrane Biol. 14, 143–176
14 Boheim, G. and Kolb, H.A. (1978) J. Membrane Biol. 38, 99–150
15 Nagaraj, R. and Balaram, P. (1981) Acc. Chem. Res. 14, 356–362
16 Irmscher, G. and Jung, G. (1977) Eur. J. Biochem. 80, 165–174
17 Ames, B.N. and Dubin, D.T. (1960) J. Biol. Chem. 235, 769–775
18 Baechinger, H.P., Eggenberger, H.P. and Haenisch, G. (1979) Rev. Sci. Instrum. 50, 1367–1372
19 Klotz, I.M. and Hunston, D.L. (1971) Biochemistry 10, 3065–3069
20 Ben-Naim, A. (1980) Hydrophobic Interactions, Ch. 9, Plenum Press, New York
21 Lewis, B.A. and Engelman, D.M. (1983) J. Mol. Biol. 166, 211–217
22 Schwarz, G. and Savko, P. (1982) Biophys. J. 39, 211–219
23 Jung, G., Dubischar, N. and Leibfritz, D. (1975) Eur. J. Biochem. 54, 395–409
24 Latorre, R., Miller, C.G. and Quay, S. (1981) Biophys. J. 36, 803–809
25 Boheim, G. (1974) J. Membrane Biol. 19, 277–303
26 Reiss-Husson, F. (1967) J. Mol. Biol. 25, 363–382