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Electrical interactions of membrane active peptides at lipid/water interfaces

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Abstract

Vital functions of biological membranes are frequently controlled by amphipathic peptides that are associated with the lipid bilayer. The extent of association is largely determined by influences encountered at the interface between the aqueous and lipid moieties, especially involving electrostatic interactions. A basic thermodynamic analysis is presented in terms of a partitioning equilibrium where the membrane is treated as a non-ideal solution of peptide molecules in a two-dimensional lipid solvent. This may then be employed to interpret experimental association isotherms (i.e. the ratio of associated peptide per lipid plotted versus the free aqueous peptide concentration) in the light of a molecular mechanism. Special emphasis is directed towards the evaluation of original titration data under most general circumstances when the association can be monitored using a suitable linear signal (preferentially an optical one). The experimental approaches as well as the merits regarding possible information about the underlying structural and functional features are discussed with pertinent practical examples.

Keywords: Association isotherms; Liposomes; Mastoparan; Partition coefficients; Salt effects; Thermodynamics

1. Introduction

Lipid bilayers may be functionally modified or activated by peptides (and larger proteins as well) that are sufficiently hydrophobic and possibly also carry electrical charges. With biological cell membranes such membrane active agents may control critical physiological processes, for instance pore formation (gating), fusion or signal transduction. The physical chemistry of the underlying molecular mechanisms has so far been widely unexplored. At any rate, the interactions being experienced at the lipid/water interface play a crucial role regarding the amount and structural state of those substrate molecules that are associated with the lipid membrane and accordingly may actually intervene in the process under consideration. In addition to amphiphilic effects diverse electrostatic interactions will be of essential significance.

For an investigation of basic aspects in the present field it would be most appropriate to choose comparatively simple model systems involving unilamellar lipid vesicles and some well characterized peptide material suspended in an electrolyte solution. First of all, one needs to know how much of the total peptide is actually associated with the vesicle membrane. It is, however, rather pointless to carry out a formal evaluation of 'binding' data using a Scatchard plot

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as sometimes is seen in the literature. This lacks the necessary foundation, namely the existence of specific and independent binding sites. Any apparent 'binding site' for a given peptide molecule would involve a greater number of lipid molecules so that overlapping and interfering with neighboring 'sites' is inevitable. The same problem has been encountered and theoretically treated for the binding of large ligands on a linear polymer composed of smaller subunits [1,2]. The two-dimensional case applicable to a lipid membrane turned out to be quite complicated mathematically [3,4]. Therefore one has to look for a more serviceable approach in the quantitative interpretation of peptide–membrane association.

We refer to the now generally accepted idea of describing a cell membrane in terms of a 'fluid-mosaic model' as proposed by Singer and Nicolson [5]. These authors have already advanced the concept of a two-dimensional solution where the lipid bilayer is the solvent and any associated protein makes up the solute. Accordingly the 'binding' of peptide and other ligand out of the aqueous moiety into the membrane may be readily considered as a partitioning process between two immiscible liquids [6]. Along such a line one may take advantage of the analogous physical–chemical treatment of ordinary mixtures, solutions and phase transitions. In the following, previous approaches of this kind are to be discussed and demonstrated in a more general context.

2. Theoretical principles

From a thermodynamic point of view the chemical potentials of the various possible molecular states of the peptide in the whole system are of foremost interest. First we envisage the monomeric peptide in the given aqueous surroundings. Its chemical potential, μ', at a concentration c (and c^0 being the unit concentration, preferentially 1 M) is generally formulated:

$$\mu' = \mu_0'^{\infty} + RT \cdot \ln(y \cdot c/c^0) \qquad (1a)$$

where $\mu_0'^{\infty}$ stands for the standard potential at virtually 'infinite' dilution in pure water extrapolated to unit concentration and the quantity y refers to the appropriate activity coefficient. The value of $\mu_0'^{\infty}$ is thus derived from the free energy of peptide hydration, while the magnitude of y accounts for any mutual interactions between individual peptide molecules and possibly other solute. Under ordinary conditions of experiments with membrane active peptides their free aqueous concentration c is so small (i.e. of the order of μM or less) that y does not depend on c itself but only on a more highly concentrated additional solute, particularly specific buffer and further electrolyte. It is primarily the electrostatic work of setting up an ionic atmosphere around charged groups of the peptide that determines the value of y. The appropriate and presumably negative work term $\mu_{el}' = RT \cdot \ln\ y$ may then be added to $\mu_0'^{\infty}$ resulting in

$$\mu' = \mu'^{\infty} + RT \cdot \ln(c/c^0) \text{ with } \mu'^{\infty} = \mu_0'^{\infty} + \mu_{el}' \qquad (1b,c)$$

which now involves a definitely salt-dependent standard potential.

For a lipid associated peptide state a chemical potential can be deduced analogously. However, because of the two-dimensional nature of the solution, the concentration variable should be properly adapted. It appears most suitable to employ the associated peptide per lipid ratio, $r = c_{as}/c_L$ (the molarities being related to the total volume of the system). The chemical potential of a lipid associated state of the peptide accordingly reads

$$\mu = \mu^{\infty} + RT \cdot \ln(\alpha \cdot r) \qquad (2a)$$

with a pertinent activity coefficient α (implying $\alpha \to 1$ for $r \to 0$) that results from solute–solute interactions in the two-dimensional solvent, i.e. between lipid associated peptide molecules. These could be of appreciable magnitude since the lipid bilayer associated peptide molecules may readily come quite near to each other, exerting repulsive or attractive forces so that $\alpha > 1$ or < 1, respectively. The standard potential in Eq. 2a could be subjected to several electrical influences depending on the structural features of the lipid bilayer and the associated peptide. This may be formulated as

$$\mu^{\infty} = \mu_0^{\infty} + \mu_{el} + (ze_0\psi_0 - pE) \cdot N_A \qquad (2b)$$

(N_A: Avogadro's constant) where the first term is due to the free energy of solvation with neighboring water and lipid (including the effect of hydrophobic

interactions). Setting up a peptide specific ionic atmosphere on the aqueous side of the interface would again cause a salt dependent contribution, denoted μ_{el}, (which should be negative). In the case of charged lipids the interface could assume an electric potential ψ_0 relative to the aqueous phase so that for a number of z elementary charge units e_0 a potential energy of $N_A z e_0 \psi_0$ must be included in μ^{∞}. Finally, we have to consider a possible permanent dipole moment of the associated peptide molecule, a component p of which may be oriented parallel to the electric field strength E in the membrane (as generated by an electric membrane potential), resulting in a free energy decrease of $N_A pE$ [7].

The 'binding' equilibrium of a peptide monomer to the lipid bilayer must be subjected to the condition that the chemical potentials of Eqs. 1 and 2 are equal. By rearranging this equation one immediately obtains the basic partitioning relation

$$r = (K_p/\alpha) \cdot c \tag{3}$$

involving a partition coefficient

$$K_p = \exp\{-\Delta\mu^{\infty}/RT\} \tag{4}$$

with $\Delta\mu^{\infty} = \mu^{\infty} - \mu'^{\infty}$ being the standard Gibbs energy change per mol upon a transition of the peptide from the aqueous to the lipid surroundings. A plot of experimental data representing r versus free peptide concentration c, the association isotherm, may be fitted by such a functional expression provided an appropriate formulation of α in terms of r is available. Electrostatic repulsion between charges on neighboring associated peptides could be a most effective factor in controlling α. Using a Gouy–Chapman model treatment the relation

$$\ln \alpha = 2z \cdot \sinh^{-1}(zbr) \tag{5}$$

has been derived for a single associated state with a net charge number of z, electrically neutral lipid and a univalent electrolyte (the fixed parameter b being dependent on the given ionic strength) [8].

The partition coefficient K_p is apparently equal to the initial slope of the association isotherm. According to Eqs. 1b and 2b it may be expressed as

$$K_p = K_p^0 \cdot \exp\{-\Delta\mu_{el}/RT\} \cdot \exp\{-ze_0\Psi_0/kT\} \cdot \exp\{pE/kT\}. \tag{6}$$

A fundamental value of the partition coefficient, K_p^0, is solely determined by the change of intrinsic solvation interactions in the lipid/water interface relative to pure water. A salt effect is induced owing to a possible difference in the electrostatic work gained when the ionic atmospheres are formed ($\Delta\mu_{el} = \mu_{el} - \mu'_{el}$). In addition, there are Boltzmann factors describing contributions to the 'binding' affinity resulting from net charges of the lipid and a permanent dipole of the associated peptide, respectively.

The present approach can be extended to the case of two or more associated peptide states as for instance other conformations and/or aggregates of monomers. The pertinent equilibria between such states may be quantitatively expressed by appropriate mass action laws based on the relevant stoichiometry and chemical potentials. An example has been given elsewhere for an apparent strong aggregation of a membrane incorporated antibiotic peptide [9].

3. Experimental methods

In practical work it will be of first concern that the amount of associated peptide is measured in one way or the other. Then the overall association isotherm, i.e. the total 'binding' ratio r versus free peptide concentration c_f, can be constructed and possibly be interpreted in terms of a partitioning equilibrium together with the appropriate mass action laws of any further molecular conversion processes that may occur in the system.

A quite useful titration approach towards r vs. c_f has been introduced some time ago [10]. It takes advantage of a specific physical signal Φ (e.g., optical absorption, circular dichroism, fluorescence emission) that depends linearly on the concentrations of the various possible molecular states of the peptide. Here an extended and most general derivation is to be given. We set

$$\Phi = \left(\sum_i m_i F_i c_i\right)_{aq} + \left(\sum_i m_i^* F_i^* c_i^*\right)_{as}.$$

The F_i, F_i^* are the pertinent coefficients characterizing the signals emitted by the individual aqueous and lipid associated peptide states, respectively; m_i, m_i^*

stand for the number of monomers and c_i, c_i^* denote the concentrations (mol per volume). This may be rewritten to read

$$\Phi = F_0 \cdot c_f + F_\infty \cdot r c_L \text{ with } F_0 = \left(\sum_i m_i F_i c_i \right) / c_f$$

$$F_\infty = \left(\sum_i m_i^* F_i^* r_i \right) / r c_L \qquad (7a,b,c)$$

where

$$c_f = \sum_i m_i c_i \quad r = \sum_i m_i^* r_i \quad (r_i = c_i^*/c_L) .$$

Now it must be emphasized that by reason of thermodynamic principles the equilibrium values of any c_i, r_i are functionally related to each other. One of them necessarily determines the whole set. This is founded on the fact that for a total of n such concentration variables $n-1$ independent equations for them will exist because of the equilibrium conditions for the particular chemical potentials (being a generalization of Eq. 3). Accordingly, for instance, the quantities F_0, F_∞ and r are definitely controlled by c_f alone (or vice versa). Owing to the mass conservation of the entire peptide concentration in the system, namely

$$c_P = r \cdot c_L + c_f \qquad (8)$$

we may then introduce a normalized signal

$$(\Phi - \Phi_0)/c_P \equiv \Delta F = \Delta F_\infty \cdot r \cdot (c_L/c_P) \qquad (9a)$$

where $\Phi_0 = F_0 \cdot c_P$ is the measured signal without added lipid and $\Delta F_\infty = F_\infty - F_0$ reflects the difference of averaged signals between the associated and free aqueous states. Now we propose to monitor the course of ΔF versus c_L/c_P in a series of titration experiments done at different c_P. From the data one may directly determine the quantity $Q = \Delta F/(c_L/c_P)$ which because of Eq. 9a can alternatively be expressed as

$$Q = \Delta F_\infty \cdot r. \qquad (9b)$$

Thus any pairs of c_L, c_P taken at the same Q (on

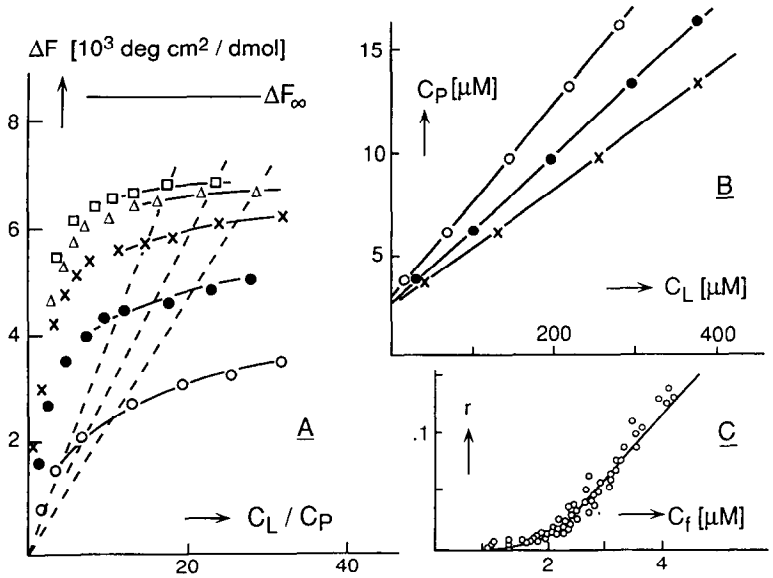

Fig. 1. Processing of titration experiments in order to establish the association isotherm of a peptide at a lipid vesicle/water interface as generally described in the text. The present data refer to alamethicin in 10 mM Tris buffer (21°C, pH 7.4) and DOPC–SUV [9]. (A) The signal ΔF (i.e. the increase of the negative molar elipticity per residue at 224 nm) for various peptide solutions with fixed concentration c_p when gradually raising the lipid concentration c_L (the c_p have been equal to 3.7, 6.2, 9.7, 13.1, 16.2 μM going upward). The initial F_0 was always equal to 4800 deg cm^2/dmol. The dashed lines (with slopes Q = 394, 300, 235 deg cm^2/dmol, respectively) make intersections on the titration curves where the c_P, c_L pairs correspond to the same values of r and c_f. (B) Linear plots of c_P vs c_L (according to Eq. 8) for the given Q. The slopes, r, result in an invariant ΔF_∞ = 8400 deg cm^2/dmol (by application of Eq. 9b). (C) Initial part of the association isotherm as evaluated from all the available titration data.

different titration curves) must fall under the same values of pertinent r and c_f so that according to Eq. 8 a plot of c_P vs. c_L must result in a straight line having a slope of r and an ordinate intercept of c_f. Once r is known, the value of ΔF_∞ can be determined by means of Eq. 9b. Having evaluated ΔF_∞ as a function of Q, the r vs. c_f curve is readily obtained using the Eqs. 8 and 9b. In simple cases ΔF_∞ and F_0 would be invariant with respect to Q (for example, if there is only one aqueous and one associated peptide state, or if the individual F_i and F_i^* in each of the two phases do not differ).

A demonstration of the method is presented in Fig. 1. The data have been taken from an investigation carried out with the pore-forming peptide alamethicin interacting with small unilamellar vesicles (SUV) made of dioleoylphosphatidylcholine (DOPC) [9]. Upon titration of an aqueous peptide solution with the liposomes substantial changes of the circular dichroism spectrum occur indicating an increase of secondary structure upon association. The negative elipticity at 224 nm has accordingly been chosen as a suitable means to monitor the partitioning process: ΔF being the increase of negative molar elipticity per amino acid residue when adding more and more lipid. In Fig. 1A a number of titration curves (ΔF vs. c_L/c_P) is given. Evidently, a straight line through the origin has a slope of $\Delta F/(c_L/c_P) = Q$, thus it meets the condition of invariant r and c_f for any intersection with a titration curve. Plotting the respective c_P vs. c_L then should result in a straight line as is in fact the case according to Fig. 1B. From the slope, r, the appropriate value of ΔF_∞ is readily obtained (Eq. 9b) so that finally the association isotherm, r vs. c_f, may be determined (Fig. 1C) from the original ΔF without any special assumptions about the 'binding' model.

4. Discussion

It has been emphasized that the 'binding' of membrane active peptides (and other similar agents) to a lipid bilayer is most reasonably described in terms of a partitioning process between the aqueous medium and the lipid moiety which is acting as a separate solvent in two dimensions. Decisive factors for the degree of membrane association are the interactions occurring at the lipid/water interface, the electrostatic ones in particular. Because of appreciable solute–solute interactions the lipid associated molecules must be considered to exist in a thermodynamically non-ideal solution state, being quantitively characterized by a specific activity coefficient. A pertinent analysis of experimental association isotherms ('binding' curves), as for instance the one shown in Fig. 1C, accordingly offers a possible means to explore structural as well as energetic conditions being encountered in the interfacial surroundings.

A rather popular measuring method makes use of a titration approach where the progress of association is monitored by a suitable linear signal Φ. Sometimes it is assumed that an apparent plateau of Φ (reached at higher c_L/c_P) does imply that all the peptide has been converted to its final associated state, thus reflecting the corresponding relative signal F_∞ which could be used to calculate the degree of association in the course of the titration experiment. This is, however, by no means a safe conclusion as is clearly demonstrated by the examples of Fig. 1A. It needs at least titrations at two sufficiently different c_P to check whether there is an obvious tendency towards a common plateau. Even then some caution is called for since it follows from Eq. 7b and c that F_0 and/or F_∞ may possibly depend on the concentrations as they gradually change upon the progress of titration (such cases are to be expected when peptide aggregates are formed with different signal intensities).

Membrane active peptides usually induce a more or less pronounced pore formation effect in a lipid bilayer. Such a function has been especially well investigated with alamethicin, an antibiotic peptide of 20 amino acid residues [11]. In order to elucidate the underlying molecular mechanism, the extent and structural state of the lipid associated material is of basic interest. This has set off some work to study the interaction of alamethicin with lipid vesicles [6,7,9,10,12]. As already indicated by the example in Fig. 1 the pertinent association isotherms exhibit an apparent strong increase of the 'binding' affinity once a certain critical concentration is exceeded. These phenomena could be quantitatively well described in terms of a model featuring a comparatively weak partitioning of monomeric peptide, being

coupled to a powerful aggregation of the associated molecules. The non-ideal nature of the system was taken into account by means of an activity coefficient derived from a kind of excluded area model. The appearance of aggregates proved to be correlated with the formation of ion conducting channels in planar lipid membranes. The observed voltage dependence of the latter event could be readily explained by the electric field effect on the partition coefficient (see Eq. 5) owing to the permanent dipole moment of incorporated alamethicin [9]. Neither the peptide nor the lipid carries a net electric charge. In compliance with this fact no salt effect was observed in the case of the lipid dimyristoylphosphatidylcholine (DMPC). However, adding salt did enhance aggregation when the likewise zwitterionic DOPC was employed, suggesting an appreciable salt sensitive dipole–dipole interaction contributing to the association affinity.

More recently very much attention has been directed to electrically charged amphipathic peptides (which show a polar as well as an apolar face when adopting a helical conformation), being composed of only natural amino acids (in contrast to alamethicin). They are considered to be highly useful models of membrane channel forming structures [13]. Melittin, the main constituent of bee venom (26 residues), is a typical and intensely explored exponent of this class [14]. In particular, it has been studied in some detail regarding its association with liposomes using the same titration approach with a circular dichroism signal that was applied to alamethicin [8,15,16]. In this case, however, no indication of an aggregation in the membrane became apparent. The association isotherms could be fitted quite satisfactorily by means of a monomer–monomer partitioning equilibrium according to Eq. 3 with a Gouy–Chapman kind activity coefficient as expressed by Eq. 5. The two adjustable parameters, the partition coefficient K_p and the charge number z, turned out to be variable depending on salt content, pH and modifications of the actual physical charge.

The general idea of the merit of such research may be pointed out in view of some results obtained for another interesting specimen of the same group of peptides: mastoparan, a wasp venom factor (composed of only 14 amino acid residues namely: $^{+}$Ile–Asn–Leu–$^{+}$Lys–Ala–Leu–Ala–Ala–Leu–Ala–$^{+}$Lys–$^{+}$Lys–Ile–Leu–NH_2). Titration could also be carried out using a circular dichroism signal [17]. Salt effects on the various parameters are compiled in Table 1. In a lipid-free solution the relevant signal F_0 turned out to be independent of concentration and added salt, possibly owing to a lack of aggregation of the aqueous monomers (in contrast to melittin where a pronounced tetramerization has been observed at higher ionic strength). However, the signal of the lipid associated state, F_∞, appears to decrease somewhat beyond a threshold value of about $Q = 150$ deg cm^2/dmol, suggesting a slight degree of aggregation at higher extent of association. The F_∞ given in Table 1 refer to a lower Q (corresponding to $r < 5 \cdot 10^{-2}$, i.e. a lipid to associated peptide ratio > 20).

Table 1
Parameters evaluated from a titration of mastoparan (three different values of c_p in the range of 6–11 μM) with DOPC vesicles monitored by the change of the negative molar elipticity per residue at 222 nm (10 mM Tris–HCl buffer, pH 7.4, 0.5 mM EDTA, 20°C)

NaCl added (M)	F_0 (deg cm^2/dmol)	F_∞ (deg cm^2/dmol)	K_p (10^3 M^{-1})	z
0.0	7400	30000	1.65	+0.95
0.02	7400	26700	3.1	+1.5
0.1	7400	23100	7.0	+1.4
0.4	7400	20200	17	+1.75

Up to about $Q = 150$ deg cm^2/dmol a concentration-invariant F_∞ (as shown) was observed. Above this value F_∞ decreased slightly.

These data reflect a striking salt effect on the secondary structure of the associated peptide. Apparently the presence of salt promotes a state with less helical content (indicated by a decrease of F_∞) where, however, the free energy of the ionic cloud evidently tends to become more favorable so that the partition coefficient, K_p, increases markedly.

There is also a change of the effective charge number, z. In the first place, it must be pointed to the fact that z is always found to be clearly smaller than the true physical charge number which should be rather above 3 for the pH in question. This peculiar phenomenon has been very generally observed in analogous cases with multicharged associated peptides. It can be attributed to a fundamental defect of the Gouy–Chapman model. The latter pre-

sumes charges being smeared over the very interface between the water and the lipid domains. Naturally, however, one deals with discrete charges which may penetrate more or less into the electrolyte and also imply excluded volume effects. Such deficiencies of the applied model result in a reduction of the apparent charge number [18]. The observed effective value of z will accordingly also be subject to changes depending on the definite structure of the associated peptide which is salt dependent as demonstrated by the data in Table 1.

Acknowledgements

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