INCORPORATION KINETICS IN A MEMBRANE, STUDIED WITH THE PORE-FORMING PEPTIDE ALAMETHICIN

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ABSTRACT The reaction of fluorescence-labeled alamethicin with unilamellar phospholipid vesicles (DOPC and DMPC) has been investigated in a stopped-flow apparatus. Clearly single exponential time functions have been observed at temperatures above the phase transition of the bilayer. This can be interpreted in terms of an essentially one-step incorporation process. The pseudo first-order forward rate is found to be quite fast, falling in a range somewhat below the diffusion controlled upper bound. The data are quantitatively very well described on the basis of a simple mechanism. This comprises diffusion of peptide into the bilayer accompanied by a more or less slower change of the secondary structure. Aggregation of the incorporated molecules at higher concentrations is indicated to be comparatively rapid.

1. INTRODUCTION

Alamethicin is an antibiotic peptide of 20 amino acid residues (1). When added to a phospholipid bilayer it forms ion conducting pores depending on voltage. This simple and well defined gating phenomenon has been extensively studied (2). Nevertheless its molecular mechanism is not fully understood so far. The pores must be aggregates of some kind, composed of a number of monomeric peptides (3).

In the present article we shall primarily report and discuss some basic physico-chemical properties of alamethicin interacting with a lipid bilayer. Their possible significance for the gating function will be indicated. However, no elaborated model of the gating mechanism is to be proposed here.

There is yet an additional objective of our paper. We feel that the various approaches and results described may be useful in many other cases where any kind of association of a ligand with a membrane is considered. That is meant most generally, comprising binding or adsorption to the surface as well as incorporation into its interior. As far as alamethicin is concerned, the available evidence strongly suggests substantial incorporation. First of all, the molecule is largely hydrophobic so that it exhibits a high tendency to enter amphiphilic solvents, such as octanol, instead of water (4). Accordingly we may expect a considerable extent of penetration into the apolar domain of a lipid bilayer rather than binding or adsorption to the polar head group region. This has been confirmed by spectroscopic techniques (5, 6) and pertinent cross-linking experiments (7).

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Very recently, the partition equilibrium of alamethicin between unilamellar lipid vesicles and an aqueous phase was quantitatively evaluated by an appropriate analysis of circular dichroism data (8, 9). The results clearly show not only incorporation into the bilayer membrane, but also the formation of larger aggregates. Since the peptide molecule can be assumed to carry a substantial dipole moment (10, 11), incorporation and aggregation will be enhanced by an electric field being applied parallel to the direction of the dipole (which implies a decrease of the free energy of the incorporated peptide). Thus the partitioning between the membrane and water phases may possibly be the essential voltage-dependent step in the molecular gating mechanism of alamethicin (8, 9). Naturally this can only be true if the transfer of peptide between these phases is sufficiently fast in line with the experimentally observed rate of pore formation.

To examine this point we have attempted to perform kinetic measurements. Unfortunately no signal was available to monitor promptly the partitioning reaction with ordinary alamethicin. Therefore we have attached a fluorescence label to the C-terminal end group. Using this derivative successful experiments have been carried out with two lipid bilayer species, working at the high end of the peptide concentration range, which is customary in conductance studies. The results reflect a well pronounced one-step association process. Its rate turns out to be rather fast, not much below the diffusion-controlled upper bound.

2. MATERIALS AND METHODS

The phospholipid species 1,2-dimyristoyl-sn-glycero-3-phosphorylcholine (DMPC) and 1,2-dioleoyl-sn-glycero-3-phosphorylcholine (DOPC) were obtained and used for the preparation of solutions of small unilamellar vesicles as described elsewhere (9). There we have also given a detailed report on the preparation and characterization of the alamethicin. Its

phenylalaninol C-terminal end group was then linked up with an anthranilate group providing a serviceable fluorescent chromophore.

The reaction between alamethicin (8.6 mg or 4.4 μmol) and isatoic anhydride (25 μmol) dissolved in 2 ml of dimethylformamide was carried out at 70°C under nitrogen flow and magnetic stirring in the presence of 4-dimethyl-amino pyridine (10 μmol) as a catalyst. Formation of the alamethicin-anthranilate (ALA-A) was followed by thin layer chromatography (TLC) (using $CHCl_3/CH_3OH/H_2O$ = 65:25:4 as the elution medium [12]), by monitoring the appearance of a new fluorescent spot under UV illumination (λ = 366 nm) with an R_f higher than that of unreacted alamethicin. After 3 h 30 μmol of isatoic anhydride and 20 μmol of 4-dimethylamino pyridine were added and the solution was slowly allowed to reach room temperature overnight. Then, the solvent was removed in vacuo and the residue resuspended in 1 ml CH_3OH, centrifuged, and washed again with 1 ml methanol. The combined supernatants were applied to a Sephadex LH 20 column (3 cm × 35 cm) in methanol. ALA-A eluted in the front peak and could be readily purified from the excess reagents with two passages through this column. Final purification by preparative TLC, followed by lyophylization from *t*-butanol, yielded 6 mg (65%) of a chromatographically pure product. Comparison between the absorption coefficient (ϵ_{340} = 5,000 M^{-1} cm^{-1}) and the molar ellipticity per residue ($[\theta]_{220}$ = −12,500 deg $dmol^{-1}$ cm^2) in methanolic solution indicates the presence of one chromophoric group per peptide chain, as expected on the basis of the known structure and reactivity of alamethicin (13).

Equilibrium fluorescence has been recorded with a recording fluorometer (model RRS 1,000; Schoeffel Instruments Corp., Westwood, NJ). The kinetic measurements were carried out in a thermostatted Durrum (Dionex Corp., Sunnyvale, CA) stopped-flow apparatus (dead time ~3 ms) equipped with two monochromators and a fluorescence detection system (as developed in our department) (14). In a typical experiment one of the syringes contained a solution of labeled alamethicin, the other one an equal volume of pure vesicle solution (pH 7.4, 10 mM Tris/HCl). Upon mixing (which implies a concentration jump of total peptide as well as of lipid to one-half) interaction of the label with the bilayer moiety leads to an increase of fluorescence intensity, which was registered as a function of time.

Electrical conductance measurements were done on planar lipid films formed from DOPC in *n*-decane (1% wt/vol) on Teflon sandwich septa with a hole of 0.16-mm diam. The hole was pretreated with 0.5% DOPC in hexane and the lipid film painted with the help of an air bubble. Alamethicin was added in microliter amounts from a methanolic stock solution directly to the aqueous phase. The electrical conductance properties of alamethicin anthranilate were found to be very similar to those of the unmodified peptide. Single pores were well resolved at 1 M KCl as well as 0.2 M KCl. The voltage dependence of the conductance increase was the same as with ordinary alamethicin, showing an *e*-fold rise every 4.4(±0.2) mV.

3. RESULTS

3.1 Equilibrium Studies

To evaluate the association equilibrium of the peptide between the bilayer and the aqueous phases we have measured the fluorescence intensity, I, as it increases when lipid vesicles are gradually added to an originally aqueous peptide solution of fluorescence intensity I_o. The relative signal $F = (I - I_o)/I_o$ in DOPC at 20°C has been determined as a function of the lipid concentration, c_L, for various values of the total peptide concentration c_p (which is kept constant in the course of titration). These data are plotted versus c_L/c_p in Fig. 1.

The measured curves clearly demonstrate an association of the peptide with the lipid bilayer. This leaves still open

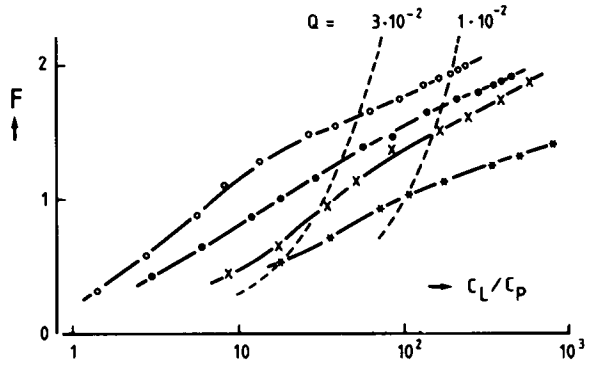

FIGURE 1 Relative increase of fluorescence emission, F, for the labeled alamethicin when titrated with DOPC vesicles at 20°C (c_L, lipid concentration). The solid curves refer to constant peptide concentrations c_P (in μM): 0.81 (○), 0.40 (●), 0.14 (×), 0.07 (*). The dashed curves represent examples of contour lines at a constant value of $Q = F \cdot (c_P/c_L)$. On such a dashed curve the associated peptide-to-lipid ratio, r, as well as the free aqueous peptide concentration, c_f, must be the same everywhere. The respective plot of c_P versus c_L then becomes a straight line according to Eq. 4 (see text in Section 3.1).

the question of adsorption or incorporation. In any case, however, it will be possible to evaluate the concentrations of the associated and free (aqueous) peptide, c_{as} and c_f, respectively. For the sake of convenience we introduce the quantity $r = c_{as}/c_L$, the associated peptide-to-lipid ratio, as an appropriate measure of the extent of association. According to thermodynamic principles, r must always be a definite function of c_f (8). To determine this association isotherm, r versus c_f, we have applied a most general procedure, which is to be developed in the following.

We allow an arbitrary number of different associated peptide states. Let F_j be the given relative signal if all the peptide is converted from the free state to a certain associated state $j(= 1, 2, \ldots)$. When a mole fraction x_j of the total amount of peptide exists in that state its contribution to the overall signal F becomes $F_j \cdot x_j$. With r_j being the corresponding peptide-to-lipid ratio we have $x_j = r_j \cdot (c_L/c_P)$ so that summing up all individual contributions readily leads to

$$F = F_\infty \cdot r \cdot (c_L/c_P), \tag{1}$$

involving an appropriate average of the individual signals, $F_1, F_2, \ldots$, namely

$$F_\infty = F_1 \cdot (r_1/r) + F_2 \cdot (r_2/r) + \cdots \tag{2}$$

We note that $Q = F \cdot (c_P/c_L)$ is a directly accessible experimental quantity which because of Eq. 1 must be subject to the relation

$$Q = F_\infty \cdot r.$$

On the other hand, mass conservation implies that

$$c_P = r \cdot c_L + c_f. \tag{4}$$

Thus r and c_f can be easily calculated from the measured Q at given c_P and c_L provided F_∞ is known.

Evidently, F_∞ becomes a constant parameter of the system if either there is only one lipid-associated state or if all the states have the same specific signal (i.e., $F_1 = F_2 = \ldots$). Under such circumstances one can apply a simplified extrapolation procedure yielding $F \rightarrow F_\infty$. This has been demonstrated recently with the circular dichroism signal of ordinary alamethicin (8, 9). However, assuming a constant F_∞ value did not work satisfactorily in the present case.

Consequently we have switched to a more generally applicable method suitable for a calculation of the amount of lipid-associated peptide. Basically it starts from the fact that F_∞ as well as r and therefore also Q (see Eq. 3) must be an explicit function of c_f even in the most complex equilibrium case. This follows from thermodynamic principles: The individual chemical potentials of the various lipid states of the peptide depend on the concentration variables $r_1, r_2, \ldots$, whereas the chemical potentials of all possible aqueous states depend on the appropriate aqueous concentrations. At equilibrium the chemical potential of any peptide state must be the same. These conditions imply a functional relationship between the r_j and c_f (see the pertinent argumentation in reference 8). Thus a given c_f determines not only the individual r_j but also the overall quantities r, F_∞, and Q. Vice versa, this means that different sets of c_P, c_L with the same Q value involve common values of r, c_f, and F_∞. Examples of contour lines with fixed Q are given in Fig. 1. If at least two c_P, c_L sets for a certain Q value have been found, the pertinent r, c_f can be evaluated using the linear relation of Eq. 4. In other words, r proves to be the slope of a plot of c_P vs. c_L taken from different titration curves at the position of equal Q.

Having processed our data in this way we obtain the association isotherm presented in Fig. 2. As was pointed out in the introduction there are good reasons to assume an incorporation of alamethicin into the bilayer. Thus we consider the curve to reflect a partitioning of the peptide between the lipid and aqueous phases. A partition coefficient, $\Gamma = r/c_f$, of $\sim 1.8 \cdot 10^5\ M^{-1}$ is applicable up to $\sim c_f = 0.25\ \mu M$. Beyond this critical concentration an apparent enhancement of Γ is observed, possibly due to a spread of aggregate formation. Anyway, we note a level of the partition coefficient that ranges about a factor of 100 above that of ordinary alamethicin (8, 9). Comparable orders of magnitude have been reported for the partition coefficients of other hydrophobic substances such as nonactin (15) and valinomycin (16).

3.2 Kinetic Experiments

The stopped-flow measurements were carried out at a series of different temperatures (15°–31°C with DOPC, 27°–39°C with DMPC). In all these cases we observed a single exponential time response (see Fig. 3). Only below the phase transition temperature (i.e., <23°C for DMPC) more complex transients of the fluorescence signal were observed. They will not be considered in the present report.

Formally our results can be readily understood in terms of a one-step association/dissociation process (17). The forward reaction, i.e., the association of the two components to form incorporated peptide, should have a second-order rate $v_{as} = k_{as} \cdot c_L \cdot c_f$ with k_{as} being the corresponding rate constant. The reverse reaction, i.e., the dissociation of incorporated molecules, is then subject to a first-order rate $v_{dis} = k_{dis} \cdot c_{in}$, where $c_{in} = r \cdot c_L$ stands for the concentration of the incorporated peptide. The overall rate equation (at a constant amount of lipid) can now be written

$$dr/dt = k_{as} \cdot c_f - k_{dis} \cdot r = k_{as} \cdot c_P - 1/\tau \cdot r, \qquad (5)$$

(t, time) where a relaxation time τ is defined by

$$1/\tau = k_{as} \cdot c_L + k_{dis}. \qquad (6)$$

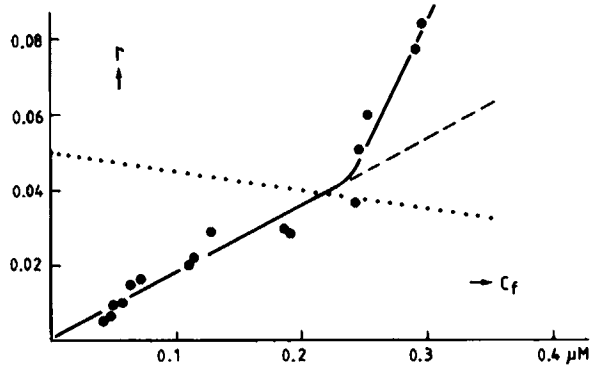

FIGURE 2 The incorporated peptide-to-lipid ratio, r, plotted versus the free aqueous peptide concentration, c_f, at equilibrium, evaluated from the data of Fig. 1. The slope of the dashed line corresponds to a partition coefficient $\Gamma = 1.8 \cdot 10^5\ M^{-1}$. The dotted staight line implies the condition of mass conservation according to Eq. 4 for a $c_P = 1\ \mu M$ and $c_L = 20\ \mu M$. Its point of intersection with the solid curve thus results in the appropriate r, c_f at equilibrium.

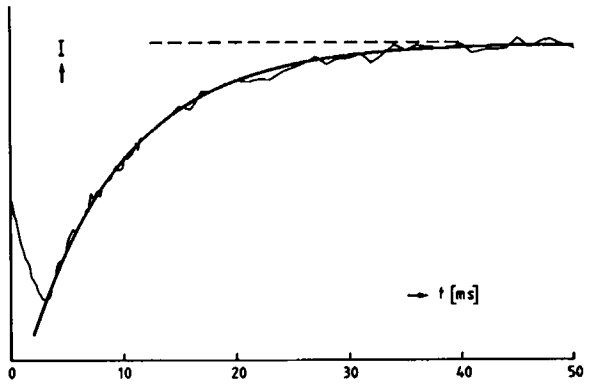

FIGURE 3 Example of a measured relaxation curve. The fluorescence intensity I (arbitrary units) is registered as a function of time after mixing the labeled alamethicin and DOPC vesicle solutions: $c_P = 2\ \mu M$, $c_L = 115\ \mu M$; 31°C. The smooth curve follows a single exponential time function with $\tau = 8.6$ ms. The initial phase where the intensity goes down falls in the dead time range of the stopped-flow apparatus.

Therefore, equilibration of r at constant c_L and c_P will be subject to a single exponential time course:

$$r = \bar{r} + (r_o - \bar{r}) \cdot \exp(-t/\tau),$$

starting from an initial value r_o (at $t = 0$). The concentration variables under equilibrium conditions, $\bar{r}$ and $\bar{c}_f$, apparently are related to each other according to

$$\bar{r}/\bar{c}_f = \Gamma = k_{as}/k_{dis}, \quad (7)$$

as is immediately derived from Eq. 5 by setting $dr/dt = 0$.

The measured relaxation time τ indeed proved to be practically independent of c_P but decreased significantly with c_L. As an example we present the detailed list of Table I which refers to DOPC vesicles at 31°C. In Fig. 4 the reciprocal τ has been plotted versus c_L in a range of moderately low c_P/c_L. As can be clearly seen, the linear relationship of Eq. 6 is in fact satisfied. From the slope we obtain a rate constant of association, $k_{as} = 9.1 \cdot 10^5$ $M^{-1}s^{-1}$. Since the experimental slope is rather well pronounced, this k_{as} is subject to little uncertainty (about a few percent). The rate constant of dissociation, k_{dis}, can be determined as the intercept on the ordinate axis. The result apparently becomes somewhat less certain, $k_{dis} \approx 9\ s^{-1}$. Then we have $k_{as}/k_{dis} = \Gamma \approx 1.0 \cdot 10^5\ M^{-1}$.

It should not be overlooked that the $1/\tau$ measured at $c_P/c_L > 0.2$ falls clearly below the straight line of Fig. 4 (see inset of Fig. 4). This can be formally ascribed to the apparent rise of Γ at larger peptide-to-lipid ratios (which is indicated for the present system in Fig. 2 but has been observed also with ordinary alamethicin). For instance, the τ measured at $c_L = 2.5\ \mu M$ would be in accordance with $k_{dis} = 2.1\ s^{-1}$, $\Gamma = 4.3 \cdot 10^5\ M^{-1}$ and a constant k_{as}.

Analogous circumstances have been encountered for the other temperatures and both kind of lipids. The rate constants, being determined by linear regression analysis of the experimental $1/\tau$ vs. c_L, are collected in Table II. Inspecting the k_{as} values we notice a decidedly smaller association rate with DMPC instead of DOPC. For either system a regular Arrhenius plot is found leading to the activation energies given. Some caution may be appropriate regarding the listed values of k_{dis} and $\Gamma = k_{as}/k_{dis}$, respectively, because of their higher level of uncertainty. For DOPC at 19°C we can, however, improve this situation by considering an independent value of $\Gamma \approx 1.8 \cdot 10^5\ M^{-1}$ as determined by the equilibrium data of Fig. 2. This is in

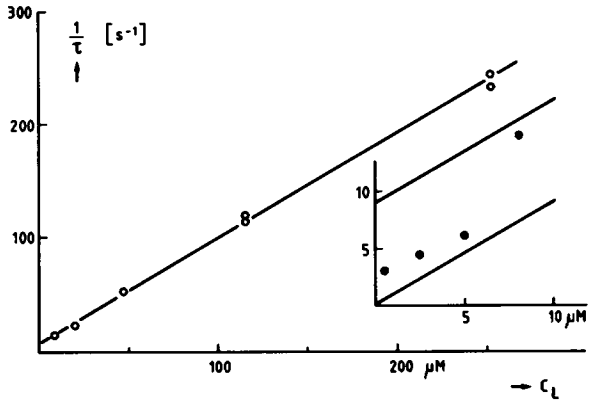

FIGURE 4 Reciprocal relaxation times, as taken from Table I, plotted versus lipid concentration in a range where the incorporated peptide appears to exist as monomeric molecules. The inset refers to small c_L, which are likely to involve aggregation: the upper straight line is retained from the main figure, the lower one corresponds to Eq. 16 with the same slope k_{as}.

TABLE I
MEASURED RELAXATION TIMES AND SUPPLEMENTARY EQUILIBRIUM PROPERTIES FOR THE STOPPED-FLOW EXPERIMENTS WITH THE FLUORESCENCE-LABELED ALAMETHICIN AND DOPC VESICLES AT 31°C

c_p	c_L	τ	$1/\tau$	c_f/c_p	r
μM	μM	ms	s^{-1}	%	10^{-2}
1	0.5	335	3.0		
1	2.5	225	4.4		
1	5	165	6.1		
1	8	67	15	≈50	≈5
1	20	44	23	33.3	3.3
1	45	19	53	18.2	1.8
1	113	8.7	115	8.1	0.81
2	115	8.6	116	8.0	1.6
1	253	4.3	233	3.8	0.38
2	253	4.1	244	3.8	0.76

The c_p and c_L refer to the conditions after mixing. The given fractions of free aqueous peptide, c_f/c_p, and associated peptide to lipid, r, are those to be expected in the final equilibrium (based on a $\Gamma = 10^5\ M^{-1}$ as given in Table II; at $c_L < 20\ \mu M$ aggregation is supposed to set in considering the results at 20°C as presented in Fig. 2).

TABLE II
PHENOMENOLOGICAL RATE CONSTANTS (ACCORDING TO EQ. 5) FOR THE INTERACTION OF THE FLUORESCENCE-LABELED ALAMETHICIN WITH PHOSPHOLIPID VESICLES

DOPC (8–250 μM)*				
T	k_{as}	$k_{as}/k_d^{\ddagger}$	k_{dis}	$\Gamma = k_{as}/k_{dis}$
°C	$10^5\ M^{-1}\ s^{-1}$		s^{-1}	$10^5\ M^{-1}$
31	9.1	0.16	9.0	1.0
27	8.6	0.17	7.6	1.1
23	7.4	0.16	7.0	1.1
19	6.3	0.15	4.3	1.5
15	5.6	0.15	1.3	4.3
DMPC (250–750 μM)§				
T	k_{as}	$k_{as}/k_d^{\ddagger}$	k_{dis}	$\Gamma = k_{as}/k_{dis}$
°C	$10^5\ M^{-1}\ s^{-1}$		s^{-1}	$10^5\ M^{-1}$
39	2.8	0.041	0.8	3.5
35	2.4	0.039	1.9	1.3
31	1.8	0.032	4.9	0.37
27	1.2	0.024	9.3	0.13

*Activation energy of k_{as} is $E_{as} = 23.3$ kJ/mol, $r_{AP} = -0.992$ is the linear correlation coefficient of the Arrhenius plot.
‡k_d is the upper bound of k_{as} determined by diffusion in the aqueous medium (see Discussion).
§Activation energy of k_{as} is $E_{as} = 55.3$ kJ/mol, $r_{AP} = -0.983$.

fact fairly close to the value of $1.5 \cdot 10^5\ M^{-1}$ in Table II. Accordingly our kinetic method of determining k_{dis} and Γ appears to be fairly reliable. The reciprocal value of k_{dis} actually represents the average lifetime of a peptide molecule associated with the bilayer. Under our conditions these lifetimes are seen to fall in the range of 0.1–1 s.

Recent equilibrium studies have shown that partitioning of ordinary alamethicin is practically independent of temperature in the case of DOPC (9). The kinetic data with our present fluorescent species indicate a moderate enhancement of Γ upon cooling. However, the results for DMPC exhibit a rather drastic effect of temperature. There is a remarkable decrease (!) of the dissociation rate at increasing temperature (formally described by a negative activation energy). This also implies a substantial increase of the partition coefficient when raising the temperature.

4. DISCUSSION

4.1 Basic Ideas

Let us first recall the obvious evidence exhibited by our kinetic experiments. There was always a single exponential time course of the fluorescence signal after mixing peptide and vesicle solutions in a stopped-flow apparatus (at temperatures above the lipid phase transition). The reciprocal relaxation times plotted versus lipid concentration followed a straight line. This implies that the underlying reaction can be formally described as a one-step association/dissociation process with rate constants k_{as} and k_{dis}, respectively.

The measured k_{as} are expected to have a diffusion controlled upper limit k_d. This k_d actually stands for the rate constant of an encounter state formation, i.e., the arrival of a "free" peptide molecule on the vesicle surface. Having reached such a state, either diffusion back into the bulk aqueous medium or conversion to the associated state will follow. Along the lines of this phenomenological picture we have calculated k_d as well as k_{-d}, the reverse rate constant of encounter state formation. Since the observed k_{as} turn out to be appreciably smaller than the respective k_d, we can determine the rate constant k_c describing the conversion of "free" to "associated" peptide on the vesicle. So far no definite assumption about the real nature of the association is needed.

Turning to the question of mechanism we argue that the peptide undergoes incorporation. In addition to the obvious translocation this must involve also a conformational transformation (because of circular dichroism evidence). We can indeed interpret our data quantitatively in terms of such a most simple scheme of intermediate reactions. The translocation rate may be calculated assuming diffusion of the molecule into the bilayer driven by the accompanying drop of free energy. Then one finds that the overall incorporation in DOPC is nearly as fast as the translocation can possibly proceed. The somewhat lower rate in DMPC, however, suggests a pronounced rate-limiting effect of the structural transition.

Most of the measurements have been done with concentrations that apparently permit the aggregation of incorporated peptide to be neglected. At larger r values where such aggregation presumably occurs (see Fig. 2) we see no additional slower relaxation step but a pronounced decrease of the measured k_{dis}. This suggests that aggregation proceeds comparatively fast.

After having outlined the general conception that we have in mind the various aspects shall now be discussed in greater detail.

4.2 Analysis of the Phenomenological Rate Constants

Our results can be interpreted in terms of a simple scheme generally applicable to the transfer of any molecule P from its aqueous ("free") state P_f to some vesicle-associated state, P_{as} (17):

$$P_f\,(+\text{ lipid}) \underset{k_{-d}}{\overset{k_d}{\rightleftharpoons}} P_{enc} \underset{k_{-c}}{\overset{k_c}{\rightleftharpoons}} P_{as},$$

Scheme I

which involves an intermediate "encounter state" P_{enc}, i.e., a molecule in its aqueous state located at a position directly on the bilayer surface. The reactions $P_f \rightleftharpoons P_{enc}$ are described in terms of a second-order rate constant k_d (considering the effect of lipid concentration) and a first-order reverse rate constant k_{-d}. These are fully determined by diffusion in the aqueous phase. The P_{enc} can undergo the actual conversion to P_{as} (k_c and k_{-c} being the respective first-order rate constants). We note that P_{enc} is a purely geometrical state of low population. Therefore it is subject to the steady state condition. This allows reformulation of Scheme I as

$$P_f\,(\vdash \text{lipid}) \underset{k_{dis}}{\overset{k_{as}}{\rightleftharpoons}} P_{as},$$

Scheme II

with the rate equation of Eq. 5 provided we set

$$k_{as} = [k_c/(k_c + k_{-d})] \cdot k_d, \tag{8a}$$

$$k_{dis} = [k_{-d}/(k_c + k_{-d})] \cdot k_{-c} \tag{8b}$$

(see reference 17 for a detailed discussion). The diffusion-controlled rate constant k_d thus represents an upper bound for k_{as}. Apparently this limit is reached in case of $k_c \gg k_{-d}$ (which applies if practically all peptide molecules arriving on the surface of the vesicle are rapidly converted). Theoretically it can be shown (17) that

$$k_d = \beta \cdot N_A \cdot D_o \cdot (A_L/R_v) \tag{9}$$

involving β, the fraction of lipid molecules that are in the outer leaflet of the bilayer; N_A, Avogadro's number; D_o, the

diffusion coefficient of P_f; A_L, the area per lipid headgroup on the bilayer surface; and R_v, the outer radius of a vesicle.

In our DOPC case we may set $A_L = 0.70$ nm^2, $R_v = 15$ nm and $\beta = 0.6$ (which corresponds to a bilayer thickness of 3 nm) (9). Considering the degree of approximation involved in our calculations, the same geometrical parameters can be used for DMPC neglecting also their temperature dependences (18). Furthermore, we have calculated D_o using the Stokes–Einstein equation with a radius $R_o = 0.85$ nm. This R_o in the aqueous state of the peptide is estimated on the basis of a partial specific volume of $\bar{v} = 0.8$ cm^3/g (10, 19) assuming an approximately spherical shape of the largely hydrophobic peptide molecule in water (the result being corroborated by the tumbling time of 0.6 ns measured for a spin-labeled alamethicin; Rizzo, V., and H. Gerke, unpublished results). At 31°C for instance, one finds that

$$k_d = 5.7 \cdot 10^6 \text{ M}^{-1} \text{ s}^{-1}$$

(with $D_o = 3.4 \cdot 10^{-6}$ cm^2/s). The k_d changes with temperature according to the temperature dependence of D_o. The corresponding activation energy for k_d is $E_d = 19.6$ kJ/mol (as calculated from handbook data of the thermal effect on the viscosity of water).

With respect to the diffusion-controlled reverse step $P_{enc} \rightarrow P_f$ the theory (17) yields

$$k_{-d} = D_o/(2R_oR_v) \tag{10}$$

assuming no energetic interaction between the vesicle and the peptide in the encounter state. At 31°C this yields a value of $k_{-d} = 1.3 \cdot 10^7$ s^{-1}, which implies an average lifetime of 75 ns for the encounter complex before the peptide would move off the bilayer surface into the bulk aqueous phase.

It ought to be emphasized at this stage that the Eqs. 8 have been derived on the condition of a stationary state of the involved diffusion process. Such a condition applies at times t (after the reaction has been started) which comply with the relation $\sqrt{\pi \cdot D_o \cdot t} \gg R_v$ (17). This is well satisfied here indeed for $t > 0.1$ ms (covering by far our measuring times).

We shall now inspect the actual data of Table II in the light of the above considerations. For the DOPC bilayers a value of $k_{as}/k_d \approx 0.16$ is obtained, nearly independent of temperature. This value indicates that only one out of six encounter complexes is actually turned over to the associated state. At 31°C for example one obtains $k_c \approx 2.5 \cdot 10^6$ s^{-1}; in other words, any peptide molecule on the bilayer can become incorporated during an average time of only ~0.4 μs.

The same values of the diffusion-controlled k_d, k_{-d} as above for DOPC must also be expected in the DMPC case. However, as reflected by the actually measured k_{as}, the k_c values are nearly one order of magnitude smaller. At 31°C we find an average conversion time of 2.3 μs for $P_{enc} \rightarrow P_{as}$.

4.3 A Possible Reaction Mechanism

The available evidence for ordinary alamethicin points to an incorporation of the peptide chain into the hydrocarbon region of a lipid bilayer (see Introduction). Reasonably the more hydrophobic NH_2-terminal end would penetrate the membrane, whereas the more hydrophilic C-terminal stays outside the apolar core. The label attached at that latter end is expected to cause no fundamental changes of the incorporation behavior.

As a most simple kinetic scheme of the actual reaction path of incorporation we propose

$$P_f\,(+\text{ lipid}) \underset{k_{-d}}{\overset{k_d}{\rightleftharpoons}} P_{enc} \underset{k_{-i}}{\overset{k_i}{\rightleftharpoons}} P'_{in} \underset{k_{-s}}{\overset{k_s}{\rightleftharpoons}} P_{in}.$$

Scheme III

This is an appropriate extension of the formal Schemes I and II. The final incorporated state P_{in} has a different conformation than the aqueous state P_f (according to circular dichroism evidence with ordinary alamethicin [9]). The overall incorporation process $P_{enc} \rightarrow P_{in}$ is therefore divided up in a pure translocation $P_{enc} \rightarrow P'_{in}$ and a structural transition $P'_{in} \rightarrow P_{in}$ (with respective forward and reverse rate constants as shown in Scheme III). The state P'_{in} refers to an incorporated peptide, which has not yet changed its secondary structure. It is assumed that not only P_{enc} but also P'_{in} is an intermediate state of low population. Thus both are subject to the steady state condition. Using the standard procedure we have calculated the rate constant k_c (see Scheme I) for the overall incorporation step $P_{enc} \rightarrow P_{in}$ as

$$k_c = \frac{\gamma' k_s}{\gamma' k_s + k_i} \cdot k_i. \tag{11}$$

This involves $\gamma' = k_i/k_{-i}$, the partition coefficient for the structurally unaltered peptide (taken on the ordinary concentration basis!).

There is no apparent activation barrier for the pure translocation of peptide from the encounter state into the lipid phase. Accordingly we propose that k_i will be determined by diffusion inside the membrane under a driving force provided by the drop of free energy (which amounts to $-kT \cdot \ln \gamma'$). The pertinent theory (17) then predicts the approximate relation

$$k_i = \frac{D_i}{2R_o x_i} \cdot \frac{\gamma'}{\gamma' - 1} \cdot \ln \gamma', \tag{12}$$

where D_i stands for the diffusion coefficient inside the bilayer and x_i measures the distance between the positions of the encounter and incorporated states.

The ordinary partition coefficient of the finally incorporated peptide is $\gamma = \Gamma/\bar{V}_L$ ($\bar{V}_L$ being the partial molar

volume of the lipid). In the present experimental cases we have $\gamma \approx 2 \cdot 10^5$ (with $\bar{V}_L = 0.8$ dm^3/mol [20], $\Gamma \approx 1.5 \cdot 10^5$ M^{-1}), at least as far as the order of magnitude is concerned. Naturally $\gamma' \ll \gamma$, whereas $\gamma' \gg 1$ is anticipated because of the largely hydrophobic properties of the peptide chain. Actually Eq. 14 proves to be rather insensitive to changes of γ' in that range. Taking yet $x_i \approx 2.5$ nm as a physically reasonable distance to be covered we can fairly well estimate

$$k_i \approx 1.5 \text{ nm}^2 \cdot D_i. \tag{13}$$

According to Eq. 11 this k_i represents the upper bound of k_c, the phenomenological conversion rate constant for the incorporation process. It applies to a fully diffusion controlled translocation and a comparatively fast structural transition (implying $\gamma' \cdot k_s \gg k_i$).

For a quantitative discussion of our data in light of the proposed mechanism we need adequate values of D_i. As a matter of fact diffusion coefficients of molecules in a membrane have been found to fall about two orders of magnitude below the corresponding values in water (21). Owing to the apparent lack of specific information for alamethicin we use the relevant data for another peptide of similar size, namely gramicidin C in DMPC and egg phosphatidylcholine (PC) (22). The reported D_i are $4.3 \cdot 10^{-8}$ cm^2/s (DMPC) and $4.9 \cdot 10^{-8}$ cm^2/s (egg PC), both at 34°C. There is a temperature-dependence equivalent to activation energies of 35 kJ/mol and 22 kJ/mol, respectively.

Adopting the egg PC data for our DOPC system, Eq. 13 yields a $k_i \approx 7 \cdot 10^6$ s^{-1} at 31°C. This is just slightly larger than our observed $k_c \approx 2.5 \cdot 10^6$ s^{-1} (see Section 4.2). We may therefore conclude that the incorporation is almost fully diffusion controlled. Such inference appears to be corroborated by the fact that our experimental activation energy of 23 kJ/mol is nearly the same as for diffusion in water (20 kJ/mol) and the lipid (22 kJ/mol). Strictly speaking, the reduction factor of $k_c/k_i \approx 0.35$ could be attributed to a weak rate-limiting effect of the structural transition. According to Eq. 11 $\gamma' \cdot k_s \approx 3.5\ 10^6$ s^{-1} would do.

For the DMPC system only slightly smaller k_i are calculated ($\approx 6 \cdot 10^6$ s^{-1} at 31°C). However, our apparent k_c range is about a factor of 25–40 below the k_i. This is consistent with a pronounced rate-limiting effect of the conformational transition (i.e., $\gamma' \cdot k_s \ll k_i$). It may be due to reduced values of γ' and/or k_s. Anyway, Eq. 11 turns to

$$k_c = \gamma' \cdot k_s. \tag{14}$$

Imagining, for example, a possible $\gamma' = 10^3$ would imply $k_s \approx 2 \cdot 10^2$ s^{-1}.

Inserting Eq. 14 in the phenomenological Eqs. 8 eventually leads to

$$k_{as} = V_o \cdot \gamma' \cdot k_s, \quad k_{dis} = (V_o/\bar{V}_L) \cdot (\gamma'/\gamma) \cdot k_s, \tag{15a, b}$$

which involves the volume factor $V_o = 2\beta \cdot N_A \cdot A_L \cdot R_o = k_d/k_{-d}$. In this case, E_{as}, the measured 55 kJ/mol activation energy of k_{as}, is not due to the activation energy of diffusion in DMPC (reported to be 35 kJ/mol as indicated before) but is composed of $\Delta H'_\gamma$, the enthalpy of incorporation of the intermediate state P'_{in} plus the activation energy of the structural transition, E_s. Nevertheless, the E_{as} is still much smaller than ΔH_γ, the overall enthalpy of incorporation (≈217 kJ/mol, as calculated from the Γ reported in Table II). This implies that formally a negative activation energy, $E_{dis} \approx \Delta H'_\gamma + E_s - \Delta H_\gamma \approx -162$ kJ/mol, must be assigned to the phenomenological rate constant of peptide dissociating out of the bilayer. In other words, the unusual temperature effect on k_{dis} is apparently due to an insufficient thermal increase of the intermediate P'_{in} population in comparison with that of the fully converted peptide in the bilayer.

Finally we shall examine the possible kinetic effect of aggregation. Above the critical concentration an extended reaction scheme is envisaged

$$P_f\,(+\text{lipid}) \underset{k_{dis}}{\overset{k_{as}}{\rightleftharpoons}} P_{in}^{(m)} \rightleftharpoons P_{in}^{(a)},$$

Scheme IV

where k_{as}, k_{dis} refer to the overall incorporation process of monomeric peptide as discussed before. In contrast to the intermediates of our previous schemes, $P_{in}^{(m)}$ cannot generally be considered to exist in very small amounts. In particular, comparable shares of the available peptide should be distributed between all the three states when one has chosen concentrations around the critical value (corresponding to $c_L \approx 10$ μM with our DOPC system as presented in Table I). Under such circumstances a slow second relaxation process must be seen in case that the aggregation step $P_{in}^{(m)} \rightarrow P_{in}^{(a)}$ proceeds at a clearly slower rate than $P_{in}^{(m)} \rightarrow P_f\,(+\text{lipid})$. This conclusion even applies if the aggregation lacks a fluorescence signal of its own (note that aggregation will necessarily be associated with an adequate reequilibration of monomer incorporation). However, we have never found indications of a second relaxation time above the one being reported. Therefore we exclude the possibility of a slow formation of aggregates.

A fairly fast aggregation, on the other hand, proves to be quite compatible with our observations. In the experimentally accessible time range a practically instantaneous monomer-aggregate equilibrium of the incorporated peptide can be assumed. Ideally the incorporated monomer concentration should remain constant once we exceed the critical concentration of the total incorporated peptide. Using standard routines of chemical relaxation kinetics (23) a relaxation time according to

$$1/\tau = k_{as} \cdot c_L \tag{16}$$

is then derived. In comparison with Eq. 6, represented as the straight line in Fig. 4, we therefore expect another

straight line involving the same slope k_{as} but a k_{dis} set equal to zero. The inset of Fig. 4 displays these alternatives in the range of very small c_L where aggregate formation is supposed to occur. Evidently the data come fairly near the lower line as they should under the condition that the monomer concentration is to a greater degree buffered owing to rapid aggregation.

The formation of aggregates can indeed be fast enough. At $r > 5 \cdot 10^{-2}$ we estimate with our previously adopted D_i values that a diffusion-controlled encounter of two incorporated peptide chains takes less than 10 μs. Against this stands a measured relaxation time of ~50 ms.

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REFERENCES

1. Rinehart Jr., K. L., J. C. Cook Jr., H. Meng, K. L. Olson, and R. C. Pandey. 1977. Mass spectrometric determination of molecular formulas for membrane-modifying antibiotics. *Nature (Lond.).* 269:832–833.
2. Hall, J. E., I. Vodyanoy, T. M. Balasubramanian, and G. R. Marshall. 1984. Alamethicin. A rich model for channel behavior. *Biophys. J.* 45:233–247.
3. Boheim, G. 1974. Statistical analysis of alamethicin channels in black lipid membranes. *J. Membr. Biol.* 19:277–303.
4. Irmscher, G., G. Bovermann, G. Boheim, and G. Jung. 1978. Trichotoxin A-40, a new membrane exciting peptide. Part A: isolation, characterization and conformation. *Biochim. Biophys. Acta.* 507:470–484.
5. Fringeli, U. P., and M. Fringeli. 1979. Pore formation in lipid membranes by alamethicin. *Proc. Natl. Acad. Sci. USA.*76:3852–3856.
6. Knoll, W. 1986. Raman-spectroscopic evidence for the incorporation of alamethicin into dimyristoylphosphatidylcholine bilayers. *Biochim. Biophys. Acta.* 863:329–331.
7. Latorre, R., C. G. Miller, and S. Quay. 1981. Voltage-dependent conductance induced by alamethicin-phospholipid conjugates in lipid bilayers. *Biophys. J.* 36:803–809.
8. Schwarz, G., S. Stankowski, and V. Rizzo. 1986. Thermodynamic analysis of incorporation and aggregation in a membrane. Application to the pore forming peptide alamethicin. *Biochim. Biophys. Acta.* 861:141–151.
9. Rizzo, V., S. Stankowski, and G. Schwarz. 1987. Alamethicin incorporation in lipid bilayers: a thermodynamic study. *Biochemistry.* 26:2751–2759.
10. Schwarz, G., and P. Savko. 1982. Structural and dipolar properties of the voltage-dependent pore former alamethicin in octanol/dioxane. *Biophys. J.* 39:211–219.
11. Yantorno, R., S. Takashima, and P. Mueller. 1982. Dipole moment of alamethicin as related to voltage-dependent conductance in lipid bilayers. *Biophys. J.* 38:105–110.
12. Melling, J., and A. I. McMullen. 1975. Separation, purification, and characterization of alamethicins produced from *Trichoderma viride.* ICS.IAMS *Proc. Sci. Council Jpn.* 5:446–447.
13. Pandey, R. C., J. C. Cook, Jr., and K. L. Rinehart, Jr. 1977. High resolution and field desorption mass spectrometry studies and revised structures of alamethicins I and II. *J. Am. Chem. Soc.* 99:8469–8483.
14. Paul, C., K. Kirschner, and G. Haenisch. 1980. Calibration of stopped-flow spectrophotometers using a two-step disulfide exchange reaction. *Anal. Biochem.* 101:442–448.
15. Szabo, G., G. Eisenman, and S. M. Ciani. 1969. The effects of the macrotetralide actin antibiotics on the electrical properties of phospholipid bilayer membranes. *J. Membr. Biol.* 1:346–382.
16. Stark, G., and R. Benz. 1971. The transport of potassium through lipid bilayer membranes by the neutral carriers valinomycin and monactin. Experimental studies to a previously proposed model. *J. Membr. Biol.* 5:133–154.
17. Schwarz, G. 1987. Basic kinetics of binding and incorporation with supramolecular aggregates. *Biophys. Chem.* 26:163–169.
18. Watts, A., D. Marsh, and P. F. Knowles. 1978. Characterization of dimyristoylphosphatidylcholine vesicles and their dimensional changes through the phase transition: molecular control of membrane morphology. *Biochemistry.* 17:1792–1801.
19. McMullen, A. I., and J. A. Stirrup. 1971. The aggregation of alamethicin. *Biochim. Biophys. Acta.* 241:807–814.
20. Reiss-Husson, F. 1967. Structure des phases liquide-crystallines de differents phospholipides, monoglycerides, sphingolipides, anhydres ou en presence d' eau. *J. Mol. Biol.* 25:363–382.
21. Clegg, R. M., and W. L. C. Vaz. 1985. Translational diffusion of proteins and lipids in artificial bilayer membranes. A comparison of experiment with theory. *In* Progress in Protein-Lipid Interactions. Vol. I. A. Watts and J. J. H. H. M. DePont, editors. Elsevier, Amsterdam. 173.
22. Tank, D. W., E. S. Wu, P. R. Meers, and W. W. Webb. 1982. Lateral diffusion of gramicidin C in phospholipid bilayers. Effects of cholesterol and high gramicidin concentration. *Biophys. J.* 40:129–135.
23. Schwarz, G. 1986. Theoretical basis of chemical relaxation. *In* Investigations of Rates and Mechanisms of Reactions. Vol. 6. 4/E, Part 2. C. F. Bernasconi, editor. John Wiley & Sons, Inc., New York. 27–139.