Membrane Channel-forming Polypeptides

AQUEOUS PHASE AGGREGATION AND MEMBRANE-MODIFYING ACTIVITY OF SYNTHETIC FLUORESCENT ALAMETHICIN FRAGMENTS*

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The short peptides, with the ease of aggregation followed by increasing salt concentration and dissociated by because of its membrane-modifying properties (1, 2). Ethanesulfonic acid; Tris, urea, suggestive of hydrophobic stabilization of the cent peptides in aqueous solutions leads to a marked blue shift and enhancement of the dansyl group emission spectrum. "Critical micelle" concentrations may be determined for the association of peptides. The longer peptides aggregate at lower concentrations than the short peptides, with the ease of aggregation following a trend similar to that for functional activity. The peptide acids aggregate only in media of high ionic strength. The peptide ester aggregates are stabilized by increasing salt concentration and dissociated by urea, suggestive of hydrophobic stabilization of the aggregates. The enthalpy of association for the monomer units has not yet been firmly established. Both higher conductance states (23-25). The site of aggregation of varied, with pentamers or hexamers being favored for the aqueous phase (23) and membrane phase (23). Estimates of the aggregation number have been varied, with pentamers or hexamers being favored for the higher conductance states (23-25). The site of aggregation of the monomer units has not yet been firmly established. Both aqueous phase (23) and membrane phase (26) aggregation have been proposed, with the aggregate in the former case inserting into the lipid membrane in response to an electric field (23).

We have earlier reported on the effects of chain length and charge of synthetic alamethicin fragments on the divalent cation permeability of unilamellar liposomes (27) and on uncoupling of oxidative phosphorylation in rat liver mitochondria (28). In order to study peptide aggregation at low concentrations, we have synthesized fluorescent peptide derivatives of alamethicin fragments. Emission parameters of the dansyl fluorophore have been shown to be sensitive to peptide aggregation (29). In this report, we show that the membrane activities of N-dansylglycyl alamethicin fragments, as measured by their ability to translocate cations in liposomes and their uncoupling of oxidative phosphorylation in mitochondria, parallel those of the parent peptide. We further examine the aqueous phase aggregation behavior of the synthetic, fluorescent alamethicin fragments to establish the effect of chain length, charge, and ionic strength on peptide association and attempt to correlate membrane-modifying activity with ease of aggregation.

The conformational flexibility of the peptide backbone (3). Closely related Aib-containing polypeptides like suzukacillin (4, 5) and trichotoxin A-40 (6) have been shown to similarly modify membrane conductance, probably by the formation of transmembrane channels (5, 6). Although most studies on alamethicin have used model lipid membrane systems, the polypeptide has been reported to increase calcium permeability of sarcoplasmic reticulum vesicles (7), uncover latent adenylate cyclase activity (8), and lyse erythrocytes (9) and leukocytes (10). It also causes fusion of lipid vesicles (11) and has been used to assay sidedness of natural membrane systems (8).

Conformational studies of model Aib-containing peptides (12-16) and synthetic fragments of alamethicin (17-20) and suzukacillin (21, 22) reveal that these polypeptides are likely to adopt highly folded 3α helical conformations, in which the helix interior is too narrow to permit passage of cations. The functional channel may be composed of an aggregate of these rigid, hydrophobic, helical polypeptides. Such a structure would contain a central aqueous core, which would span the lipid bilayer. Studies of the electrical properties of alamethicin-modified bilayer lipid membranes confirm that the functional channel does contain an aggregate of alamethicin monomers (23). Estimates of the aggregation number have been varied, with pentamers or hexamers being favored for the higher conductance states (23-25). The site of aggregation of the monomer units has not yet been firmly established. Both aqueous phase (23) and membrane phase (26) aggregation have been proposed, with the aggregate in the former case inserting into the lipid membrane in response to an electric field (23).

Ac-Aib-Pro-Aib-Ala-Ala-Gln-Aib-Val-Aib-
Gly-Leu-Aib-Pro-Val-Aib-Ala-Glu-Gln-Pbiol

Alamethicin (I), a 20-residue polypeptide antibiotic produced by Trichoderma viride, has been extensively studied because of its membrane-modifying properties (1, 2).

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The abbreviations used are: Aib, α-aminoisobutyric acid; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; D-(1-n)-OBzs, N-dansylglycyl peptide benzyl ester of the peptide consisting of the first n residues in I; D-(1-n)-OH, N-dansylglycyl peptide acid of the peptide consisting of the first n residues in I; HEPES, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; Tris, tris(hydroxymethyl)methylamine; cmc, critical micelle concentration; Z, benzylxycarbonyl; EPC, egg phosphatidylcholine.

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THEORY

The aqueous phase aggregation behavior of hydrophobic peptides is analogous to micellar association of amphipathic molecules like detergents and phospholipids (30). It is convenient to analyze the results obtained in terms of models used in studies of micellar aggregation.

Tanford (31) has estimated the change in chemical potential on micellization to be

\[ \mu_{\text{mic}}^\text{W} - \mu_{\text{w}}^\text{W} = \frac{m - 1}{m} \text{RT} \ln \text{cmc} + \frac{\text{RT}}{m} \ln (\sigma) \]

where the subscripts mic and W refer to micellar and aqueous phases, respectively, \( m \) is the average aggregation number, \( f_w \) is the activity coefficient of the hydrated monomer, and \( \sigma \) is a constant that lies between 1 and 100.

In the case of alamethicin fragments, the cmc values have been found to be in the micromolar range (see "Results"), where \( f_w \) will be ~1. Since \( \Delta G = \mu_{\text{mic}}^\text{W} - \mu_{\text{w}}^\text{W} \), Equation 1 may be rewritten as

\[ \Delta G = \frac{m - 1}{m} \text{RT} \ln \text{cmc} + \frac{\text{RT}}{m} \ln (\sigma) \]

The enthalpy change \( \Delta H \) is given by

\[ \Delta H = \frac{d \Delta G}{dT} = \frac{m - 1}{m} \frac{\text{d ln cmc}}{1/T} + \frac{R \text{d ln } \sigma}{\text{d ln cmc}} \frac{1}{1/T} \]

\[ + \frac{\text{d ln cmc}}{\text{d ln } \sigma} \frac{1}{1/T} \]

\( \sigma \) is a constant and \( m \) is not likely to be sensitive to temperature. Hence,

\[ \Delta H = \frac{m - 1}{m} \frac{\text{d ln cmc}}{1/T} \]

Determination of the temperature dependence of cmc values can, therefore, yield \( \Delta H \). A plot of \( \ln \text{cmc versus } 1/T \) should be linear if the dependence of \( \Delta H \) on temperature is small, i.e., the heat capacity of the system is low. The slope gives \( \Delta H / m - 1 \).

MATERIALS AND METHODS

All peptides were synthesized by solution phase procedures. Illustrative procedures have been described for alamethicin fragments (32). Boc-Gly was used at the NH2 terminus to facilitate dansylation of the amino group. Fluorescent peptides were purified by column chromatography on silica gel. The peptides were shown to be homogeneous by thin layer chromatography and were characterized by 270 MHz 'H NMR. The peptides used in this study are abbreviated as D-(1-n)-OH. Egg phosphatidylcholine, Sephadex G-50 (coarse), chloroform, methanol, benzyl ester function, and n is the residue number in the alamethicin sequence.

Chemical Co. All other chemicals were of analytical grade. Fluorescence spectrometer operated in the ratio mode with 5-nm excitation and emission band pass unless otherwise mentioned.

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FIG. 1. Cation-translocating activity of alamethicin fragments. Time-dependent increases in chlortetracycline-Ca2+ fluorescence in response to the addition of peptides. Peptides were added just prior to the start of the recording. Peptides and concentrations used are marked in figure. Time scales: (A) top time scale for alamethicin, lower time scale for all other peptides; (B) lower time scale for D-1-10-OBz, upper time scale for all other peptides. Note that the traces in B have been recorded at a higher sensitivity than those in A. Time \( t = 0 \) is at the start of the trace.

Comparison of membrane-modifying activity with ease of aggregation of fluorescent alamethicin fragments

Cation-translocating ability assayed by liposome technique and expressed as initial slope for 5 \( \mu \)M peptide. Uncoupling activity expressed as per cent reduction in RCI by 2.8 \( \mu \)M peptide. Ease of aggregation expressed as critical micelle concentration. All procedures described under "Materials and Methods."

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Liposome transport (initial slope for 5 ( \mu )M peptide)</th>
<th>Uncoupling activity (% re-duction in RCI by 2.8 ( \mu )M peptide)</th>
<th>Ease of aggregation (cmc)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-1-17-OBz</td>
<td>1.9</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>D-1-17-OH</td>
<td>0.5</td>
<td>44</td>
<td>&gt;50</td>
</tr>
<tr>
<td>D-1-13-OBz</td>
<td>0.7</td>
<td>90</td>
<td>3</td>
</tr>
<tr>
<td>D-1-13-OH</td>
<td>0.0</td>
<td>30</td>
<td>&gt;50</td>
</tr>
<tr>
<td>D-1-10-OBz</td>
<td>0</td>
<td>30</td>
<td>&gt;50</td>
</tr>
<tr>
<td>D-1-6-OBz</td>
<td>0</td>
<td>30</td>
<td>&gt;50</td>
</tr>
</tbody>
</table>

a At 15 \( \mu \)M.

b At 20 \( \mu \)M.

Uncoupling of Oxidative Phosphorylation—Mitochondria were isolated from the livers of adult, male rats by the method described (34). Respiratory rates of the mitochondria were measured on a Gilson model K-ICT-C oxygraph fitted with a Clark oxygen electrode at 30 °C in a medium containing 53 mM sucrose, 2.1 mM EDTA, 7.1 mM MgCl2, 110 mM Tri-HCl, and 21 mM potassium phosphate, pH 7.4, in a total volume of 1.4 ml. Succinate (18 mM) was used as substrate. Peptide in ethanol was added to state 4 mitochondria, such that no more than 5 \( \mu \)l of solution were added. Five \( \mu \)l of ethanol had no detectable effect on the respiration of mitochondria. Due to the hydrophobicity of the peptides, the cell had to be rinsed with mitochondria between experiments to remove the last traces of uncoupler (28). Protein was estimated by the biuret method (35).

Aggregation-Disaggregation Studies—Dansylated peptides in methanol were added to aqueous solutions at various concentrations and emission spectra recorded, with \( \lambda_e = 333 \) nm. A period of 5 min was allowed to elapse between measurements following each addition. The alcohol concentration was kept below 1%. The dilution arms of the curve were generated by sequential dilution from the highest concentration used in the aggregation titration. The time course of the disaggregation process was followed by rapidly diluting aggregated peptide solutions to double their volume and recording spectra at intervals of time. Variable temperature studies were carried out using a thermostatted cell assembly. Temperature was maintained constant
RESULTS

\[ \text{Ca}^{2+} \text{ Translocation in Liposomes—Ca}^{2+} \text{ influx into chlorotetracycline-containing liposomes results in the formation of highly fluorescent chlorotetracycline-Ca}^{2+} \text{ complexes \cite{27, 36}. A rise in fluorescence emission with time after addition of ionophore may, therefore, be used to monitor the ion-translocating ability of the peptides, as shown earlier \cite{27}. Fig. 1 compares the time dependences of the increase in fluorescence of chlorotetracycline entrapped in liposomes following addition of dansylated and parent peptides. A convenient parameter for quantitatively assessing the activities of different peptides is the initial slope of the fluorescence increase, immediately following peptide addition. Values of the initial slopes for the fluorescent fragments are presented in Table I. The activities of the N-dansylglycyl peptides compare well with those of the benzyloxy carbonyl-protected parent peptides. The sequence of ionophore activity for the dansylated peptides is D-1-17-OBz > D-1-13-OBz - D-1-17-OBz > D-1-13-OBz. D-1-10-OBz by guest, on December 22, 2010www.jbc.org Downloaded from
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The activities of the labeled and parent peptides are comparable. The percentage reduction in the respiratory control index may be used to compare the activities of the various peptides. These values for the fluorescent fragments are summarized in Table I. The sequence of uncoupling activity for the dansylated peptides is D-1-17-OBz > D-1-13-OBz > D-1-17-OH > D-1-13-OH. D-1-10-OBz and smaller fragments are inactive. A similar sequence was obtained earlier for the parent peptides (28).

The inset to Fig. 2 shows the variation in $\phi_{1/2}$ (concentration for half-maximal stimulation of state 4 respiration) for Z-1-17-OMe, with the concentration of mitochondria used. Up to 2 mg/ml of mitochondrial protein $\phi_{1/2}$ is essentially independent of mitochondrial concentration. The similarities in the activity of parent and fluorescent peptides as monitored by the liposomal cation transport and uncoupling of oxidative phosphorylation assays suggest that introduction of the N-dansylglycyl group does not significantly affect the membrane-modifying properties of the peptides. The fluorescent fragments may, therefore, serve as useful models for further studies of peptide aggregation.

Aggregation of Dansylated Peptides—Fig. 3 shows emission spectra of D-1-17-OBz as a function of peptide concentration. At concentrations greater than 6 $\mu$M D-1-17-OBz, the

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**FIG. 6.** Effect of urea on peptide aggregates. Emission spectra of fluorescent alamethicin derivatives, excited at 333 nm, as a function of urea concentration. Urea concentrations indicated against the traces. Insets, variations of $R = F_{\infty}/F_{\infty}$ with urea concentration. A, 50 $\mu$M D-1-10-OBz; B, 20 $\mu$M D-1-17-OBz.

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**FIG. 7.** Effect of NaCl on peptide aggregation. A, variation of $R = F_{\infty}/F_{\infty}$ with concentration of D-1-17-OH in 3 M NaCl (——O) or 1 M NaCl (——O). B, variation of $R$ with NaCl concentration for 9 $\mu$M D-1-10-OBz. C, emission spectra of D-1-10-OBz (15 $\mu$M), excited at 333 nm, as a function of NaCl concentration. NaCl concentration (molar M) marked on the spectra. D, log-log plot of the variation of cmc of D-1-10-OBz as a function of NaCl concentration.
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emission maximum shifts to lower wavelength with an enhancement in intensity. Peptide association can thus be easily monitored as in the case of emerimicin fragments (29). The ratio (R) of emission intensities at 490 nm to that at 550 nm (F<sub>490</sub>/F<sub>550</sub>) is taken as an index of aggregation and plotted in Figs. 4 and 5 for all of the labeled peptides. The critical aggregation or critical micelle concentrations determined for the various peptides are listed in Table 1. The facility of aggregation follows the sequence D-1-13-OBz > D-1-17-OBz > D-1-10-OBz, while D-1-6-OBz and the acids did not aggregate in salt-free aqueous media.

Sequential dilutions of the peptide from the aggregated state failed to yield reversible aggregation curves, as shown in Figs. 4 and 5. This hysteresis was observed for all of the peptides that associated under these conditions. Rapid dilution of aggregated peptides to one-half of their concentration yielded aggregates that dissociated over a time scale of several hours. Insets to Figs. 4 and 5 show the time course of this disaggregation process for D-1-17-OBz and D-1-10-OBz, respectively. The dissociation process could not be fitted to a single exponential decay (Figs. 4 and 5), suggesting that it is not a first order process. Fig. 6 shows the effect of urea on D-1-10-OBz and D-1-17-OBz at constant peptide concentration. Urea disaggregates the peptides and the process is essentially complete at a concentration of 3 M. Fig. 7 shows the effect of NaCl on the aggregation behavior of the 17-residue acid (D-1-17-OBz) and ester (D-1-17-OBz). Increasing the salt concentration promotes aggregation of both peptides with D-1-17-OBz aggregating only in 3 M NaCl. Fig. 7 demonstrates that D-1-17-OBz is almost completely aggregated by 3 M NaCl, even the minimal detectable concentration of either peptide was associated, with an emission centered at 490 nm. A study of the effect of NaCl concentration on the cmc of D-1-10-OBz establishes a linear relationship for the log-log plot as shown in Fig. 7. Similar results are obtained with C<sub>4</sub>Cl<sub>4</sub>.

Thermodynamics of Association—Fig. 8 shows the effect of temperature on the emission spectra of D-1-17-OBz and D-1-10-OBz. Raising the temperature leads to disaggregation of the peptides, i.e. ΔH is negative for the association process. Van't Hoff plots were constructed for the association of both peptides by determining cmc as a function of temperature (see "Theory"). The results are summarized in the insets to Fig. 8. The enthalpies obtained, assuming an aggregation number of 6, are -1.4 kcal mol<sup>-1</sup> and -3.1 kcal mol<sup>-1</sup> for D-1-17-OBz and D-1-10-OBz, respectively.

DISCUSSION

The peptides examined in this study consist almost entirely of hydrophobic amino acids. The only polar residue in D-1-10-OBz and D-1-17-OBz is Gln-7. It would, therefore, be anticipated that these peptides may associate in aqueous solution in order to minimize the apolar surface exposed to water. The formation of aggregates in the aqueous phase by these peptides is reminiscent of micelle formation by amphi-}

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Fig. 9. Model of peptide aggregates. Circles represent cross-sections through aggregates of 30 helical polypeptides. The thick line segment represents the polar face of the helices and the thin line segments the completely apolar faces. Note that in the hexameric aggregate exposure of the apolar faces to external (bulk) water is less than in the trimeric aggregate at the expense of an included (interstitial) aqueous core.

have been postulated at the membrane water interface (23). Such an aggregate would contain a fairly large central aqueous core (Fig. 9). Attempts to determine the size of the aggregates formed by the fluorescent peptides, using Sephadex gel filtration, were unsuccessful. Gel filtration runs in 4 M urea yielded symmetrical peaks corresponding to the monomer. Runs in 2 M NaCl, which should promote aggregation, resulted in precipitation of the peptides on the column. In the absence of salt or urea, broad, poorly characterized bands are obtained. As the gel filtration experiment takes a minimum of 2 h to conduct, extensive dissociation of the aggregates due to dilution can occur. Thus, the broad bands in the elution profile are indicative of monomer-aggregate equilibrium. Aggregation number of alamethicin has been estimated by sedimentation studies to be around 16 at ionic strength 0.2 M (42). The $\Delta H$ for aggregation becomes less negative with increasing chain length. This is consistent with the results of studies on detergent micelles which establish that $\Delta H$ for association increases with the length of the apolar segment (43).

Hysteresis has been observed for the aggregation-disaggregation process (Figs. 4 and 5). The rate of disaggregation is nonexponential, indicating that the equilibria involved are complex. Formation of transmembrane channels is probably cooperative, considering the sigmoidal character of the concentration dependence of membrane activity (27, 44). Cooperativity in the membrane phase can be treated by the Ising model in two dimensions. This predicts that for cooperativity parameters less than a critical value, metastable states will exist, leading to hysteresis effects (45). Interesting time-dependent changes in circular dichroism spectra of alamethicin have been reported in organic and aqueous solvent systems (46).

The peptides studied can be ordered in a sequence of decreasing membrane activity. Such sequences constructed for liposomal cation transport and uncoupling of oxidative phosphorylation are found to be identical, indicating that both events have similar structural requirements of peptide chain length and charge. It is thus reasonable to conclude that the same process underlies both events, viz., the formation of transmembrane channels. Since membrane-modifying activity increases with chain length and is attenuated by the presence of a negative charge in the acids, it appears that peptide aggregation may be the decisive step in the formation of functional channels. Table I shows that for the peptide esters, membrane-modifying activity correlates well with the ease of aggregation, as reflected by the cmc values. It may be noted that the rate of formation of transmembrane channels is dependent on two factors, the formation of aqueous phase aggregates and their insertion into the membrane. At low concentrations, the lower the cmc, the greater the amount of aggregated species likely to be present. The correlation demonstrated in Table I between membrane-modifying activity and ease of aggregation indicates that the concentration of aggregated species dominates the rate of formation of transmembrane channels.

Aggregation of the peptide acids is not observed up to 50 $\mu$M in salt-free aqueous media, but membrane activity of D-1-17-OH is detected at much lower concentrations (Table I). However, acids do aggregate at high ionic strength (Fig. 7). Estimates of the concentration of cations in the Stern layer of sodium dodecyl sulfate micelles range from 1 to 3 M (47) and are likely to be in the same range at membrane surfaces also. Furthermore, experimental protocol requires injection of aliquots of concentrated peptide solutions to the test system. The rates of disaggregation are very much slower than the insertion of peptide aggregates into the membrane (Figs. 1, 4, and 5). Thus, the "memory effect" in peptide aggregates may also be of relevance in explaining the observation of membrane-modifying activity in D-1-17-OH at concentrations below its cmc in salt-free solution.

While ease of aggregation correlates well with functional activity, aggregation itself is not a sufficient criterion for activity. For example, D-1-10-OBz aggregates at 30 $\mu$M but is inactive at that concentration. This is not surprising since a minimum chain length would be necessary for the formation of transmembrane channels. Earlier studies have suggested that a length of approximately 13 residues is the minimum required for the detection of membrane activity (27, 28).

The results described in this report establish that membrane channel-forming hydrophobic peptides aggregate in aqueous solution and that membrane-modifying activity correlates well with the ease of aggregation. Peptide association is favored in media of high ionic strength. This is particularly relevant since peptide acids aggregate only at high ionic strength and the ionic concentrations in the Stern layer of micelles and surfaces of membranes are likely to be high. The results of the present study favor a model for membrane channel formation, which involves the insertion of a preformed aggregate into the lipid bilayer (23).

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REFERENCES

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