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FLUORESCENT ALAMETHICIN FRAGMENTS

A STUDY OF MEMBRANE ACTIVITY AND AQUEOUS PHASE AGGREGATION

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The linear polypeptide antibiotic alamethicin is known to form channels in artificial lipid membranes. Synthetic 13- and 17-residue alamethicin fragments, labelled with a fluorescent dansyl group at the N-terminus, have been shown to translocate divalent cations across phospholipid membranes and to uncouple oxidative phosphorylation in rat liver mitochondria, in a manner analogous to the parent peptides. From studies of the aqueous phase aggregation behavior of the peptides, as well as their interaction with rat liver mitochondria, it is concluded that the interaction of the peptides with membranes is a complex process, probably involving both aqueous and membrane phase aggregation.

Alamethicin (I), a 20-residue polypeptide

1 5 10
Ac-Aib-Pro-Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-Gly-

15 20
Leu-Aib-Pro-Val-Aib-Aib-Glu-Gln-Phol

(I)

isolated from *Trichoderma viride* is known to form channels in artificial bilayer membranes [1–3]. A number of related α-aminoisobutyric acid (Aib)-containing polypeptides, such as suzukacillin [4], emerimicins [3], antiamoebins [3], hypelcins [5] and trichotoxin A-40 [6], also form transmembrane channels. Alamethicin is one of the smallest molecules known to form gateable channels [7], a feature that has stimulated considerable interest in its conformation [8,9] and membrane activity [7,10]. A major goal of these studies is to relate molecular conformation to channel-forming ability. We reported earlier on the effects of chain length and charge of synthetic alamethicin fragments on the divalent cation permeability of unilamellar phospholipid vesicles [11] and the uncoupling of oxidative phosphorylation in rat liver mitochondria [12]. Structural studies favour a largely rod-like 3_{10} helical conformation for these channel formers [9]. Functional channels must then be built up by aggregation of these cylindrical, hydrophobic structures. In principle, channel formation could occur by aqueous phase aggregation followind by insertion into the membrane [10] or by aggregation in the membrane phase [13]. It was therefore pertinent to examine the aqueous phase aggregation behavior of alamethicin fragments in an attempt to correlate ease of aggregation with channel-forming ability. We have chosen to use fluorescent peptide derivatives to monitor the aggregation process [14]. In this report we establish that the *N*-dansylglycyl derivatives of synthetic alamethicin fragments are active as divalent cation ionophores in liposomes and also uncouple oxidative phosphorylation in mitochondria. Chain length and charge effects, on both activities, noted for the fluor-

Abbreviations Aib, α-aminoisobutyric acid, Z, benzyloxycarbonyl; OBz, benzyl ester, Boc, *t*-butyloxycarbonyl; dansyl, 1-dimethylaminonaphthalene-5-sulfonyl; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

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escent peptides are similar to those observed for the parent peptides. It is shown that for uncharged esters the facility of aggregation parallels effectiveness as an ion channel. For the corresponding acids aggregation is detectable only in media of high ionic strength.

Experimental

All peptides were synthesized by solution phase procedures, as described in the synthesis of alamethicin I [15]. The 1–6, 1–10, 1–13 and 1–17 alamethicin fragments (see (I) for sequence) were prepared, with Boc-Gly and benzyl ester (OBz) groups at the amino and carboxyl terminals. Dansylglycyl-1–17-benzyl ester (dansylglycyl-1–17-OBz) and the corresponding shorter fragments dansylglycyl-1–6-OBz, dansylglycyl-1–10-OBz and dansylglycyl-1–13-OBz were prepared by removal of the Boc group with hydrochloride/ethyl acetate, followed by treatment with dansylchloride and triethylamine in chloroform. The fluorescent peptide esters were purified by column chromatography on silica gel and checked for homogeneity by TLC. The peptide acids dansylglycyl-(1–*n*)-OH, where $n = 6, 10, 13$ and 17, were obtained by saponification in methanol/sodium hydroxide. All peptides were homogeneous by TLC and yielded satisfactory 270-MHz ^{1}H-NMR

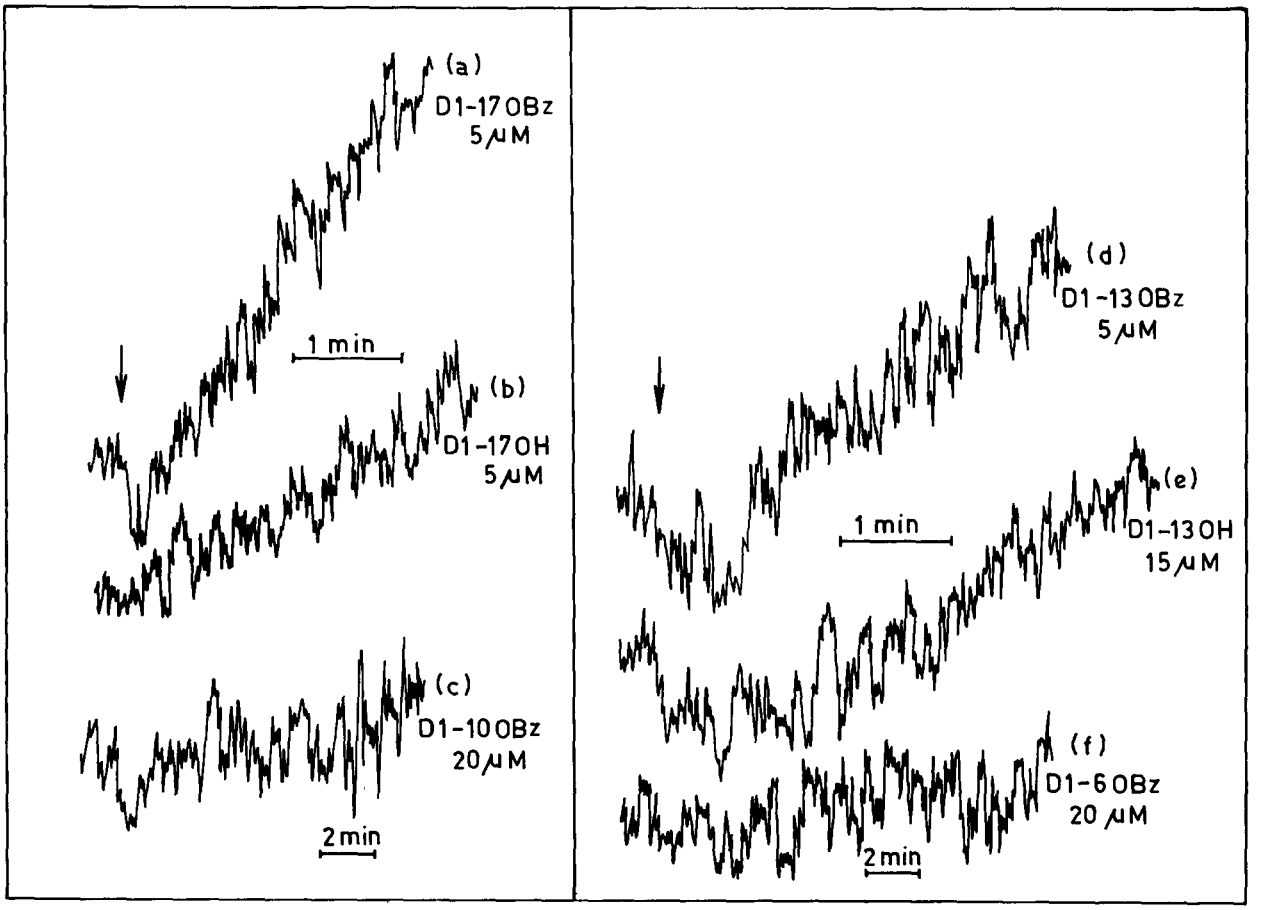

Fig. 1. Time-dependent changes in the intensity of chlortetracycline Ca^{2+} fluorescence on addition of peptides. *Y* axis, fluorescence, arbitrary units *X* axis, time scale as indicated in the figure, top scale for 13- and 17-residue peptides, bottom scale for 6- and 10-residue peptides. Arrow indicates addition of peptide. Lipid, 200 μg, chlortetracycline, 25 μM, Ca^{2+}_{ext}, 1 mM. D, dansylglycyl.

spectra. Detailed synthetic procedures will be reported elsewhere.

Egg phosphatidylcholine, HEPES, Sephadex G-50, chlortetracycline and ADP were from Sigma Chem. Co. All other chemicals were of analytical grade. Cation translocation across phospholipid membranes was followed as previously described, by a fluorescence technique using chlortetracycline in small unilamellar liposomes [11]. Mitochondria were isolated from the livers of adult male rats by the method of Johnson and Lardy [16]. Respiratory rates of mitochondria were measured as previously described [12]. Protein concentration was estimated by the biuret method [17]. Fluorescence spectra were recorded on a Perkin-Elmer Model MPF-44A fluorescence spectrometer, operated in the ratio mode with 5 nm excitation and emission band pass (10 nm for polarization experiments).

Results and Discussion

The emission characteristics of the dansyl group have been shown to be sensitive to aggregation of short, labelled fragments of emerimicin [14]. In this study *N*-dansylglycine was used as the amino terminal label, since direct dansylation of the terminal Aib group proved difficult. The membrane activity of the fluorescent alamethicin fragments was assayed by monitoring the liposomal cation transport and uncoupling activity in mitochondria induced by the peptides. Fig. 1 shows the effect of dansylated alamethicin fragments on the intensity of chlortetra-

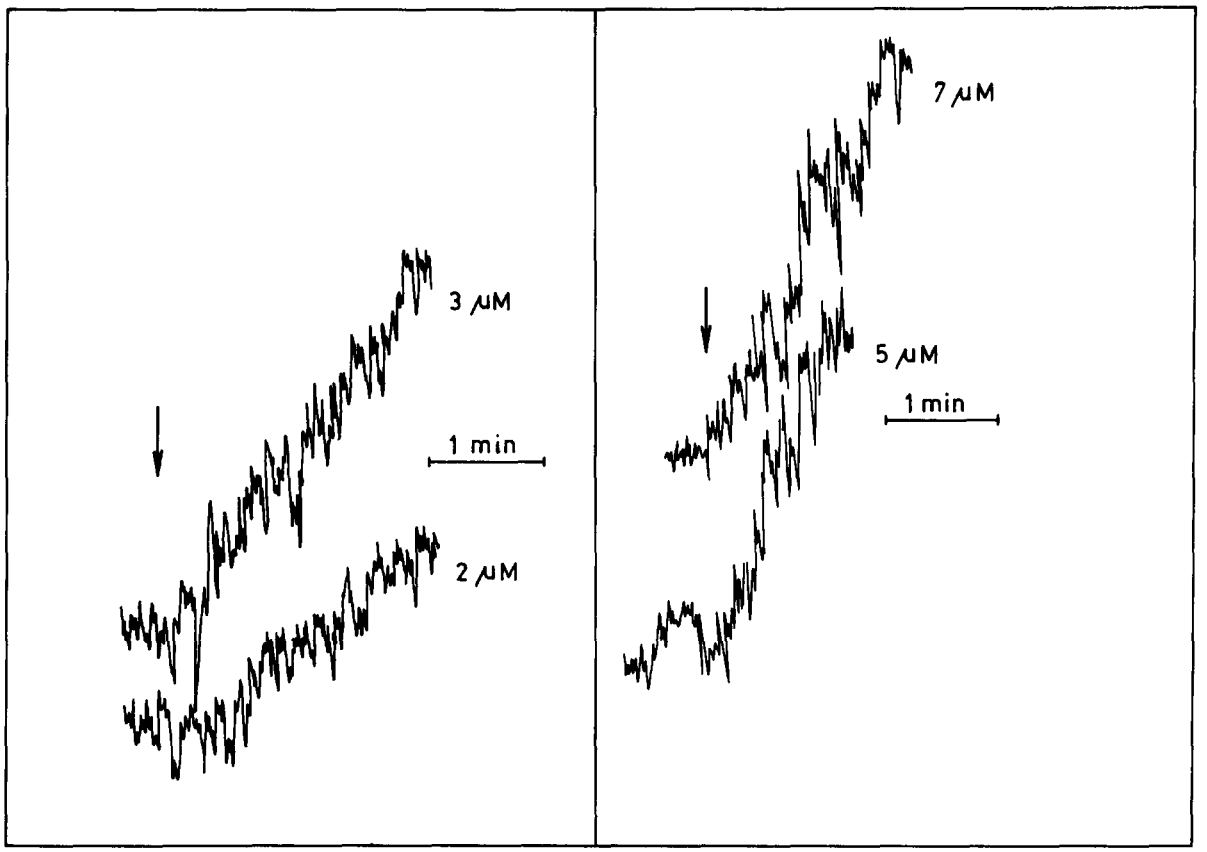

Fig 2 Time-dependent changes in the intensity of chlortetracycline Ca^{2+} fluorescence on addition of varying concentrations of dansylglycyl-1–17-OBz; concentrations indicated in the figure. Arrows indicates addition of peptide Conditions as in Fig 1

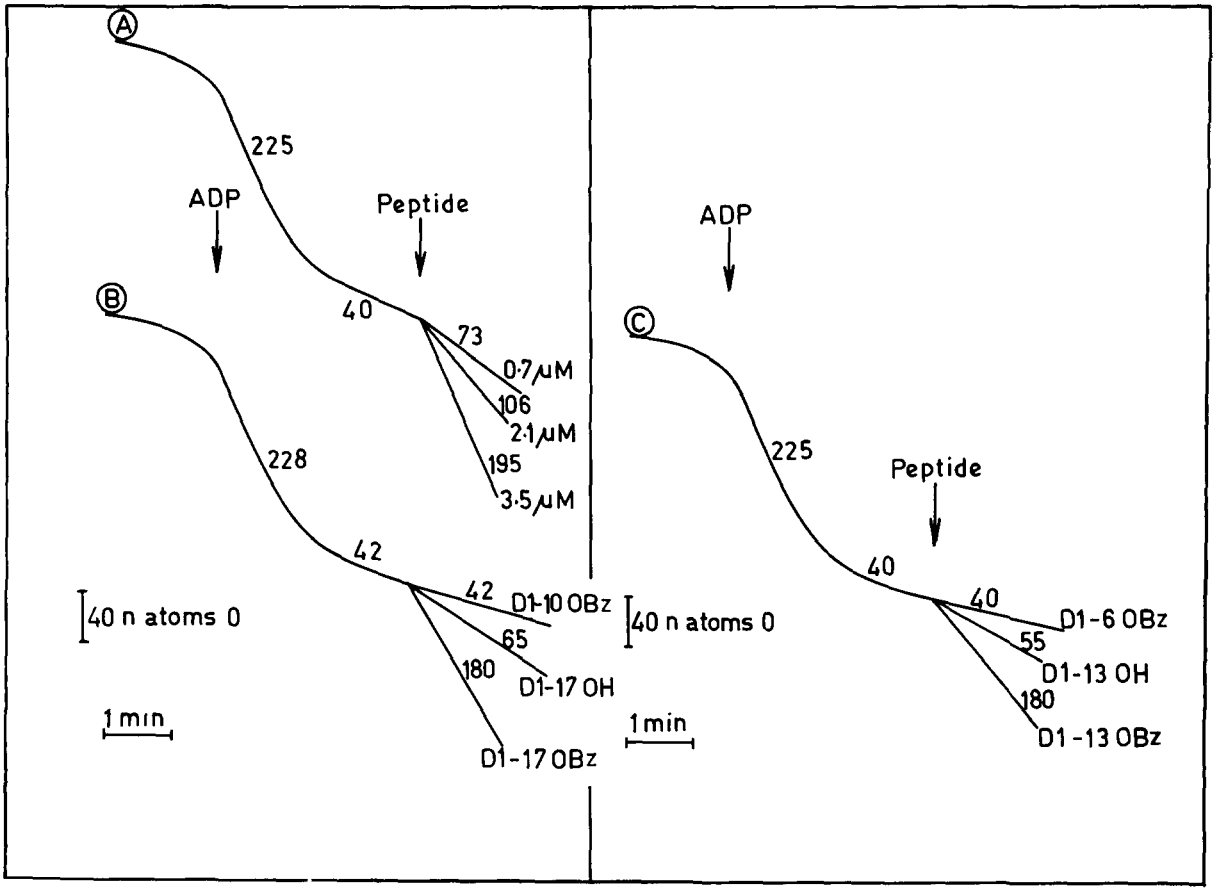

Fig. 3. Effect of peptides on oxygen consumption by rat liver mitochondria. Medium comprised 53 mM sucrose/2.1 mM EDTA/ 7 1 mM $MgCl_2$/110 mM Tris-HCl/21 mM potassium phosphate (pH 7.4)/18 mM succinate/1 mg mitochondria in a volume of 1.4 ml 200 nmol ADP were added at the indicated point. Numbers against the curves indicate oxygen consumption in natoms O/min per mg protein. A, dansylglycyl-1–17-OBz, concentrations as indicated; B and C, peptides added at 2 8 μM (except dansylglycyl-1–6-OBz and dansylglycyl-1–10-OBz, added at 20 μM;

cycline Ca^{2+} fluorescence in unilamellar liposomes. A rise in chlortetracycline Ca^{2+} emission intensity reflects the influx of Ca^{2+} into the liposomes, mediated by the ionophore. The initial slope of the curve is a measure of the ionophoretic activity [11]. The following observations were made. (i) a minimum chain length of 13 residues is necessary for activity; (ii) the esters are more active than the acids; (iii) the inhibitory effect of the negative charge for the acids is less pronounced in the case of the 17-residue fragment as compared to the 13-residue peptide; (iv) the longer fragments conduct more efficiently than the shorter ones. These results are analogous to those obtained with the parent peptides where the sequence of cation translocating activity is Ac-1–20-OBz ≈ alamethicin > Z-1–17-OMe > Z-1–17-OH ≈ Ac-1–17-OMe > Z-1–13-OMe, with Z-1–13-OH and smaller fragments being inactive [11]. It is interesting that dansylglycyl-1–13-OH is moderately active in contrast to Z-1–13-OH. This may be due to the additional glycyl residue or a consequence of the bulkier dansyl group at the amino terminus. Fig. 2 shows the concentration dependence of dansylglycyl-1–17-OBz on ion translocating activity. It can be seen that the peptide is active even at 2 μM and that increasing concentration leads to increased ion translocation, presumably by the formation of more channels.

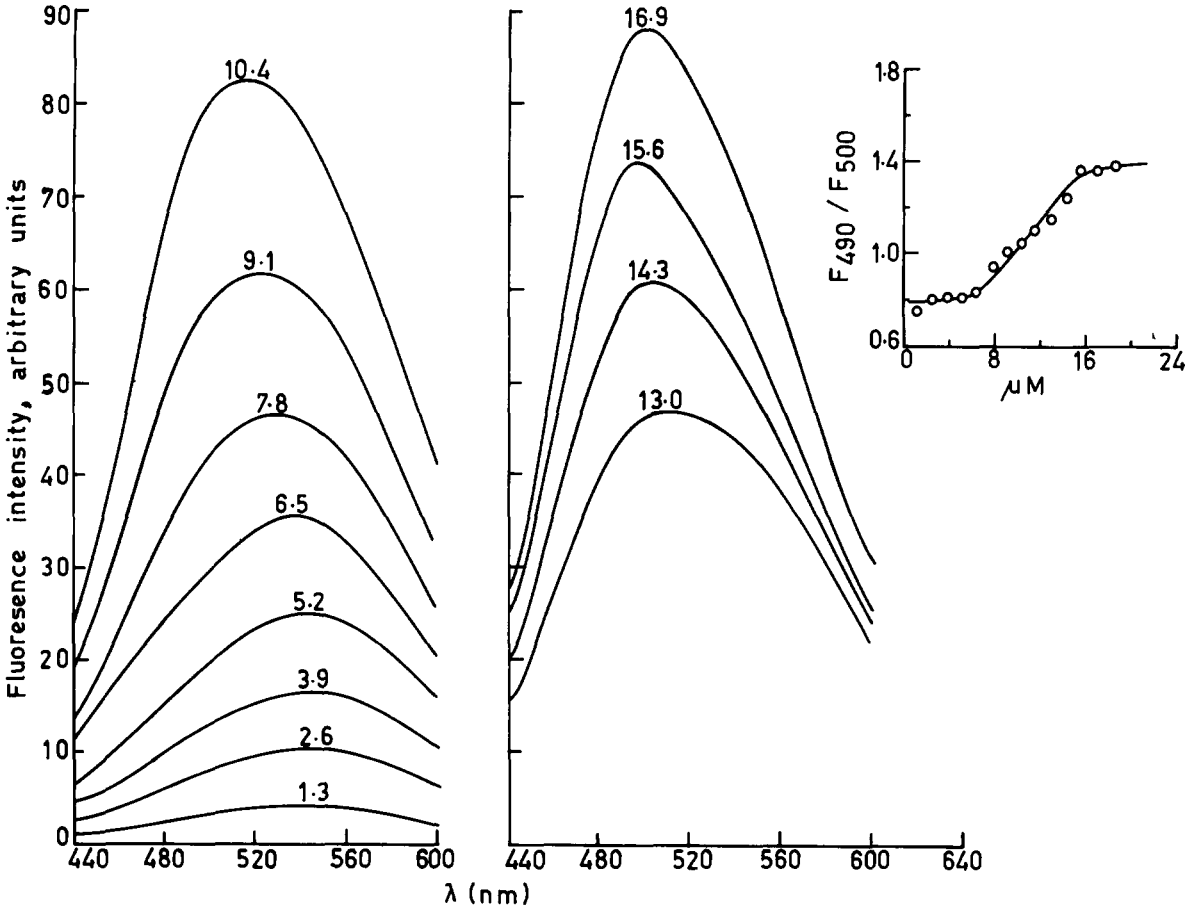

Fig. 4 Emission spectra of dansylglycyl-1–17-OBz excited at 330 nm as a function of concentration. Concentration in μM indicated on the traces. Inset variation of F_{490}/F_{550} with concentration.

Fig. 3 shows the effect of dansylated peptides on oxygen consumption by rat liver mitochondria. The sequence of uncoupling effectiveness is dansylglycyl-1–17-OBz ≈ dansylglycyl-1–13-OBz > dansylglycyl-1–17-OH > dansylglycyl-1–13-OH, with peptides less than 13 residues long showing no activity. The concentration at which half-maximal activity was obtained ($\phi_{1/2}$) was 1 μM for dansylglycyl-1–17-OBz. The activity sequence obtained earlier [12] for the parent peptides is Ac-1–20-OBz ≈ alamethicin > Z-1–20-OBz ≈ Z-1–17-OMe > Ac-1–17-OMe ~ Z-1–17-OH > Z-1–13-OMe. It may be noted that, both in the case of liposomal conductance and uncoupling activity, dansylglycyl-1–13-OBz behaves anomalously as compared to the parent peptide.

Fig. 4 shows the emission spectra of dansylglycyl-1–17-OBz (λ_{ex} = 333 nm) as a function of peptide concentration. At low concentrations the emission peak is centred at 550 nm, while at high concentrations it is centred at 490 nm. We have earlier shown that the ratio of the emission intensities at 490 and 550 nm can be used to monitor the aggregation of model hydrophobic peptides [14]. The inset to Fig. 4 shows the data treated in this manner for dansylglycyl-1–17-OBz. A clear titration curve is obtained, from which a 'critical micelle concentration' can be derived. The facility of aggregation of the peptides was found to be dansylglycyl-1–13-OBz > dansylglycyl-1–17-OBz > dansylglycyl-1–10-OBz, while no aggregation could be detected for dansyl-

glycyl-1–17-OH, dansylglycyl-1–13-OH and dansylglycyl-1–6-OBz in salt-free aqueous solution. The acids did, however, aggregate in 3 M NaCl. Further, increase in ionic strength reduced the critical aggregation concentration for the peptide esters. It is of interest to note that the ionic strength in the Stern layer of micelles [18] and presumably membrane surfaces is in the region 1–3 M. This would suggest that even the peptide acids could aggregate at the membrane interface, at the concentrations used in the assays for their activity.

In an attempt to determine whether enhanced translocation activity observed for the longer peptides was due to increased binding to the membrane, binding isotherms for the interaction of the peptides dansylglycyl-1–10-OBz, dansylglycyl-1–13-OBz and dansylglycyl-1–17-OBz with rat liver mitochondria were constructed. Relatively little enhancement in fluorescence intensity was observed on interaction with mitochondria for the peptide esters. However, changes in the polarization of fluorescence were observed. For all three peptides in solution the polarization $p = 0$, while a limiting value of $p = 0.12–0.15$ were obtained in the presence of mitochondria. Information about binding constants and stoichiometries may be obtained from polarization data [19]. It was found, however, that treatment of the data by either Scatchard [20] or Zierler [21] analysis failed to yield binding parameters, since the plots showed no possibility whatsoever of linear correlations. Since we have observed aggregation behavior in aqueous media for the peptide esters over the same concentration range as used for the binding studies, it is not surprising that little intensity enhancement was observed in the presence of mitochondria. Transfer of the dansyl fluorophore from an aggregated phase to the membrane may not result in a significant change of environment. Further, as both aqueous and membrane phase aggregation are expected to occur concurrently with binding, it is unlikely that either binding model (which assume fixed binding sites and invariant ligands) will adequately describe the interaction process.

The above results establish that dansylated alamethicin fragments, 13 or more residues long, are effective as divalent cation ionophores and in uncoupling mitochondria. Both these activities are related to the ability of the peptides to aggregate in the aqueous phase. However, aggregation is by no means a sufficient criterion for formation of functional channels. For example, dansylglycyl-1–10-OBz aggregates under 30 μM even in the absence of added salt but fails to translocate cations or uncouple mitochondria. Further, the high concentrations required for aggregating the acids is not reflected in their functional abilities. This, together with the inadequacy of conventional binding models, indicates that the interaction of these peptides with membranes is a complex process, probably involving both aqueous and membrane phase aggregation. Fluorescent, membrane-active alamethicin derivatives should prove useful in probing various aspects of peptide-lipid and peptide-peptide interactions. A detailed, quantitative study of the aggregation of these peptides will be presented, separately.

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