TRANSMEMBRANE DISTRIBUTION OF GRAMICIDIN BY TRYPTOPHAN ENERGY TRANSFER

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Determination of the tertiary structure of the physiological form of membrane proteins is one of the most challenging problems of structural biology. A partial solution to the general problem has been proposed recently (Kleinfeld, 1985; Kleinfeld and Lukacovic, 1985) and in the work described here we have applied this method to gramicidin. In this method the spatial distribution of tryptophan (Trp) residues is determined by measuring energy transfer between Trp and the *n*-(9-anthroyloxy) (*n*-AO) fatty acid membrane probes. The measured energy transfer efficiencies (T) are analyzed by a Monte Carlo procedure to obtain the coordinates of the Trp projected on a plane perpendicular to the surface of the membrane. The measurements can be done using low concentrations of the probe and conditions in which physiological alterations may be detected.

Gramicidin D was reconstituted into small unilamellar vesicles of dimyristoyl phosphatidylcholine by probe sonication. During this and in all subsequent procedures the temperature of the vesicles was maintained above 30°C. Incorporation of the AO probes in the vesicle membranes was accomplished by adding an ethanolic solution (<2% by volume) of the probe to the preformed vesicles.

To investigate whether non-Forster mechanisms are involved in the interaction of the AO probes and the gramicidin Trp (Haigh et al., 1979), we examined the effect of gramicidin on the ground and excited state properties of the AO probes as well as AO on Trp absorption in gramicidin. AO absorption spectra, quantum yields, and lifetimes were found to be independent of gramicidin, in vesicles prepared with between 0.5 and 3 mole % gramicidin. Trp absorption was also independent of AO over a range of 0.5 to 20 % probe. Thus, in the preparation used in this study, Trp quenching in the presence of the AO probes is due to Forster type transfer.

Energy transfer measurements were performed with eight different *n*-AO probes (n = 2, 3, 6, 7, 9, 10, 12, and 16) at probe-to-lipid mole fractions between 0.005 and 0.05. Fluorescence spectra of the Trp and AO emission (excitation at 290) were accumulated for each probe (see Kleinfeld and Lukacovic (1985) for details of the experimental procedures). T values were evaluated from the quenching of the Trp intensities and the sensitized AO emission. Results of studies in which gramicidin is present at >1.7 mole % are shown in Fig. 1 and exhibit a gradual rise in T as the probe position approaches the center of the bilayer.

A Monte Carlo analysis (Kleinfeld, 1985) was used to determine the Trp spatial distribution from the measured T values. Briefly, the coordinates of N Trp (where N is the number of fluorescing Trp/peptide) are chosen at random within a region circumscribed by a cylinder representing the peptide. The coordinates of each Trp are used to calculate T values for each of the eight AO probes. The values for the N Trp are averaged and these calculated values are compared with the measured values by computing the weighted mean square differences (R^2). This process is repeated until minimum R^2 values are obtained. The sets of coordinates corresponding to a minimum in R^2 are not unique. To represent the Trp distribution and to reflect this lack of uniqueness, the frequency at which a given set of coordinates occurs is determined from the pool of satisfactory fits. This frequency distribution is plotted to obtain a Trp density map as shown in Fig. 2.

The Monte Carlo search of the results shown in Fig. 1 was performed for protein radii between 6 and 20 Å and best fits were obtained for the smallest radius (6 Å) although differences in fit quality were not sufficient to exclude 8 and 10 Å. Assumptions regarding the distribution of quantum yields among the four Trp in each peptide

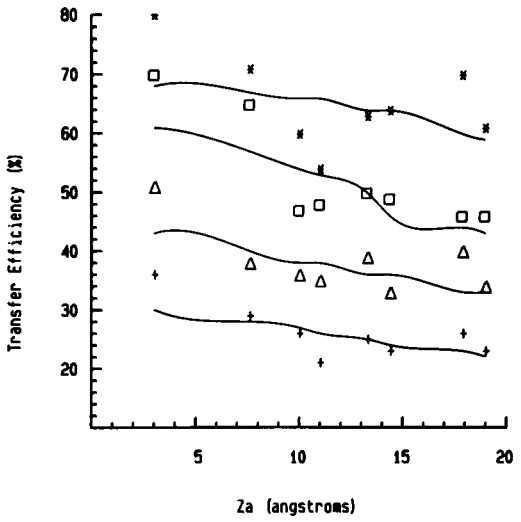

FIGURE 1 Energy transfer efficiencies between gramicidin Trp and the *n*-AO fatty acid probes. T values are plotted against the depth of the AO moiety (Za). The Za values measured relative to the center of the bilayer were obtained from a variety of experimental and molecular model considerations (Kleinfeld, 1985). Measurements, denoted by the symbols, were carried out at four different AO surface densities (in pmol/cm^2) and gramicidin:lipid mole ratios of 0.033 or 0.017; + = 6.0 and 0.033, Δ = 9.6 and 0.017, □ = 14.3 and 0.033, * = 24.7 and 0.017. The measurements were done at 34°C using phospholipid concentrations of 500 μM. The solid curves are the best fits from the Monte Carlo search to each set of transfer efficiencies.

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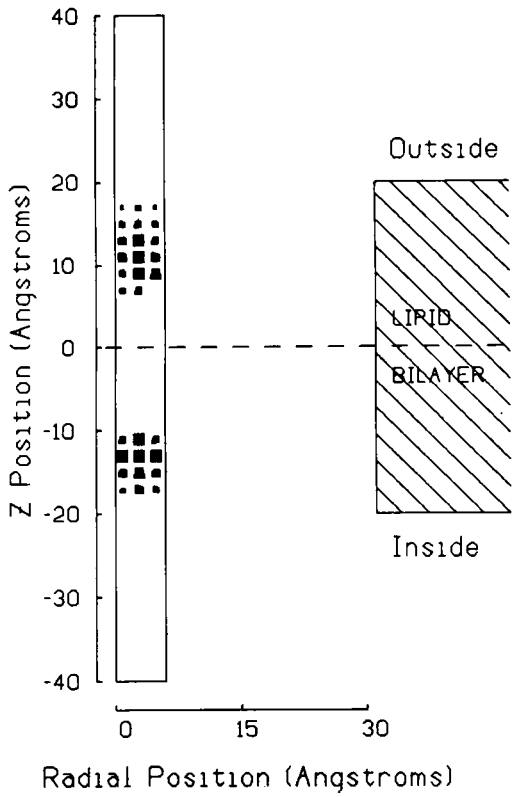

FIGURE 2 Trp density map. This map was generated after 5×10^5 iterations by distributing four Trp, each with a quantum yield of 0.22, within a cylinder of length 80 Å. The search was performed on the 9.6 pmol/cm^2 data set with a radial parameter of 6 Å. Similar maps were found for the other data sets. A two-dimensional projection is displayed since we have assumed that the probes are uniformly distributed about the cylinder and therefore the calculated transfer efficiencies are independent of the azimuthal coordinates.

do, however, dramatically affect the fit quality. (Although gramicidin D is a mixture of 85% A, with four Trp and 15% B and C with three Trp each, we have assumed that all peptides have four Trp.) In the present analysis we have varied the number of fluorescing Trp but have assumed that each has the same quantum yield; equal to the measured quantum yield for the peptide (0.4 at 34°C) divided by the number of fluorescing Trp. R^2 values were 10-fold smaller if only two Trp were assumed to be fluorescent as compared to all four. Thus the Monte Carlo analysis predicts that most of the fluorescence is due to two or fewer Trp. We have also found, by *N*-bromosuccinimide oxidation of the indole ring, that one Trp is responsible for 70% of the fluorescence.

Optimal fits to the experimental T values obtained for two fluorescing Trp and a radius of 6 Å are shown as the solid curves in Fig. 1. The corresponding density map for one of these fits is shown in Fig. 2. These results indicate that the most likely locations of the fluorescing Trp in gramicidin correspond to two groups of equal density at 14 ± 2 Å about the center of the bilayer. No assumption about the dimer nature has been used in the analysis, except that twice the single peptide number of fluorescing Trp were used in the search (i.e., instead of 1–4Trp, we used 2–8 Trp).

The Trp locations determined by this study impose severe constraints on the possible forms of the dimer. For a 6.3 β helix (Urry et al., 1971) the distance between residues is 1.1 Å. Thus a model in which the carboxyl termini are joined at the center of the bilayer predicts an average Trp position of 4.4 Å. A dimer in which the NH_2-termini are coupled predicts 13.2 Å for the average Trp locations. The NH_2-terminus to NH_2-terminus (N-N) model, which is the configuration determined by NMR (Weinstein et al., 1980), is, therefore, confirmed by the present results. Molecular models based on the 6.3 helix predict the Trp located at the 9 and 15 positions form a sandwich (Mazet et al., 1984) which, if this complex self-quenches, could account for the observed quantum yield heterogeneity. The average (unweighted) location of the Trp at position 11 and 13 is 13.2 Å, in remarkable agreement with the 14 ± 2 Å location observed from the energy transfer measurements.

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REFERENCES

Haigh, E. A., K. R. Thulborn, and W. H. Sawyer. 1979. Comparison of fluorescence energy-transfer and quenching methods to establish the position and orientation of components within the transverse plane of the lipid bilayer. Application to the gramicidin A-bilayer interaction. *Biochemistry.* 18:3525–3532.

Kleinfeld, A. M. 1985. Tryptophan imaging of membrane proteins. *Biochemistry.* 24:1874–1882.

Kleinfeld, A. M., and M. L. Lukacovic. 1985. Energy-transfer study of chytochrome b_5 using the anthroyloxy fatty acid membrane probes. *Biochemistry.* 24:1883–1890.

Mazet, J. L., O. S. Anderson, and R. E. Koeppe. 1984. Single-channel studies on linear gramicidins with altered amino acid sequences. A comparison of phenylalanine, tryptophane, and tyrosine substitutions at positions I and II. *Biophys. J.* 45:263–276.

Urry, D. W. 1971. The gramicidin A transmembrane channel: a proposed $\pi_{(L,D)}$ helix. *Proc. Natl. Acad. Sci. USA.* 68:672–676.

Weinstein, S., B. A. Wallace, E. R. Blout, J. S. Morrow, and W. Veatch. 1979. Conformation of gramicidin A channel in phospholipid vesicles: a ^{13}C and ^{19}F nuclear magnetic resonance study. *Proc. Natl. Acad. Sci. USA.* 76:4230–4234.