Effect of Salt and Membrane Fluidity on Fluorophore Motions of a Gramicidin C Derivative†

Qiuke Teng, Roger E. Koeppe II, and Suzanne F. Scarlata*,‡

Department of Medicine and Physiology and Biophysics, Cornell University Medical College, F-231, 1300 York Avenue, New York, New York 10021, and Department of Chemistry and Biochemistry, University of Arkansas, Fayetteville, Arkansas 72701

Received January 30, 1991; Revised Manuscript Received June 3, 1991

ABSTRACT: We have used fluorescence spectroscopy to investigate the effect of salt and membrane fluidity on the rotational motion of a 5-(dimethylamino)naphthalene-1-sulfonyl (dansyl) derivative of gramicidin C (dansyl-gC) in dimyristoylphosphatidylcholine bilayers, under conditions where the peptide is a formyl-NH to formyl-NH terminal dimer, and in octyl glucoside micelles, where the peptide is an intertwined helical dimer. Energy-transfer experiments showed no changes in either conformation or dimer aggregation under the conditions explored here (15–40 °C, 1–350 bar, 0–0.33 M Mg^{2+}, and 0–1 M Na^+). The addition of permeable (Na^+) or nonpermeable (Mg^{2+}) ions did not affect the temperature or pressure behavior of dansyl rotation. However, fluorescence lifetime measurements indicated an increase in solvent accessibility in the presence of sodium. In bilayers, the temperature dependence of the fluorescence polarization and lifetime shows strong interactions between the dansyl residue and the peptide, and at no time did the dansyl motions become solvent controlled as has been observed for aqueous solvent peptides [Scarlata, S. F., Rholam, M., & Weber, G. (1984) *Biochemistry 23*, 6789]. In micelles, the change in rotational motion with temperature followed solvent expansion, showing that in this case the dansyl residue does not associate extensively with the peptide. Our results indicate that because of the extensive coupling between the dansyl residue and the rest of the peptide, membrane fluidity does not play a major role in controlling side-chain motions.

The coupling of small local motions of amino acid side chains in proteins is thought to be necessary to bring about conformational changes (Careri et al., 1979). While these motions have been studied in aqueous soluble proteins [e.g., reviews of Gurd and Rothberg (1979) and Karplus and McCammon (1981)], the side-chain dynamics of integral membrane proteins have not yet been investigated. Since membrane proteins are subjected to such high lateral viscosities compared to their aqueous soluble counterparts (Radda & Smith, 1970; Cone, 1972; Rudy & Gitler, 1972; Shinitzsky & Barenholtz, 1978), their dynamics may be influenced by the membrane fluidity. At viscosities on the order of the lateral viscosity of a membrane, the side-chain motions of aqueous soluble peptides and proteins are so damped that they no longer interact with the neighboring amino acids in their subdomain and are uncoupled (Scarlata et al., 1984; Rholam et al., 1984). It is thus conceivable that the viscosity of a membrane may serve to regulate the function of integral membrane proteins by damping and undamping local motions. The activity of some membrane proteins does indeed decrease with increasing membrane viscosity brought about by high hydrostatic pressure (Varga et al., 1986; Dreyfus et al., 1988). However, this may not be a general rule. The integral membrane protein, UDP-glucuronyl transferase shows a marked increase in activity with high pressure, which appears to be due to a change in the oligomerization state of the protein (Dannenberg et al., 1990). Thus, the effects of fluidity on the local motions of membrane proteins may be masked by changes in protein conformation or aggregation.

We describe here a system in which the effect of membrane viscosity on the small-scale motions of side-chain residues of an integral membrane peptide can be readily isolated. The protein is a derivative of gramicidin. Gramicidins have the general sequence (Gross & Witkop, 1965)

HCO-L-Val-Gly-L-Ala-D-Leu-L-Ala-
D-Val-L-Val-D-Val-L-Trp-D-Leu-L-
X-D-Leu-L-Trp-D-Leu-L-Trp-$NHCH_2$-CH_2OH

where X = Trp, Phe, or Tyr at the approximate ratio of 7:1:2 and where Val-1 is sometimes replaced by Ile. Gramicidin D is a mixture of these forms [see Wallace (1990) for a general review]. This peptide, produced by *Bacillus brevis* during sporulation, is thought to regulate gene expression through binding to RNA polymerase or superhelical DNA or both (Fisher & Blumenthal, 1982; Bohg & Ristow, 1986). However, in membranes gramicidin forms channels specific for monovalent cations and has become a model system to study ion transport [for a review, see Andersen (1984)]. While much progress has been made in our understanding of the mechanism of transport, the structural changes that must occur to allow ion passage remain elusive. In hydrated bilayers whose hydrocarbon chains consist of 16 methylene groups or less, gramicidin is an N to N-terminal dimer as proposed by Urry (1971). In organic solvents and anhydrous bilayers the peptide forms an intertwined helix (Veatch et al., 1974). In both structures, the side-chain groups point out into solvent, while ion movement is through the center of the helix.

Recently, one of us attempted to determine the effects of fluidity on the side-chain rotations of the tryptophan residues of gramidicin by fluorescence polarization (Scarlata, 1988). However, the direct effects of fluidity were hampered by changes in intramolecular aromatic ring stacking. In this study, we have covalently attached a fluorescent label [5-(dimethylamino)naphthalene-1-sulfonyl or dansyl][1] on the

† This work was supported by NIH Grant GM39924 to S.F.S.

‡ Present address: Dept. of Physics and Biophysics, SUNY at Stony Brook, Stony Brook, NY 11794-8661.

[1] Abbreviations: dansyl, 5-(dimethylamino)naphthalene-1-sulfonyl; dansyl-gC, [5-(dimethylamino)naphthalene-1-sulfonyl]gramicidin C; 11AU, 11-(9-anthroyloxy)undecanoic acid; 2-, 6-, or 9-AS, 2-, 6-, or 9-(9-anthroyloxy)stearic acid; DMPC, dimyristoylphosphatidylcholine; suvs, small unilamellar vesicles; OG, octyl glucoside.

0006-2960/91/0430-7984$02.50/0

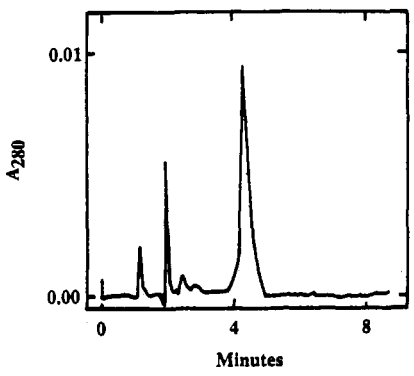

FIGURE 1: HPLC chromatogram of dansyl-gC on a 4.6 × 250 mm column of Zorbax-C8, eluted with 88% methanol at 1.5 mL/min. The solvent injection peak occurs at 2 min, with the earlier peak being a solvent impurity. The two small peaks directly after the solvent are from gramicidins C and A.

tyrosine-11 analogue of gramicidin. The functional properties of this peptide are nearly identical with those of the unlabeled peptide (Veatch et al., 1975), indicating that, in DMPC bilayers, the peptide also adapts the N to N-terminal dimer conformation. This derivative, termed dansyl-gC, offers several advantages: First, it allows for the study of fluidity effects on a single identifiable site whose motions can be observed by fluorescence polarization. Second, because tryptophan residues can transfer energy to dansyl when they are within a certain distance (Wang et al., 1988), we can determine whether dimer aggregation and possibly gross conformational changes occur under certain conditions. The former behavior would increase the number of tryptophan donors to the dansyl residue, while the latter would most likely change the distance between these residues. Third, the fluorescence lifetime of dansyl is sensitive to the dielectric of the solvent (Royer et al., 1989). Thus, by monitoring changes in lifetime with fluidity or upon the addition of monovalent (permeable) or divalent (nonpermeable) ions, we can determine whether the presence of ions in the channel changes the location of dansyl in the membrane.

We will vary membrane fluidity by changes in temperature or hydrostatic pressure. Changes in viscosity brought about by temperature reflect those due to both kinetic energy and intermolecular distances while changes by pressure only perturb the latter. Although pressure techniques have not yet been used to study the dynamics of proteins and especially not membrane proteins, the changes in bilayer properties brought about by compression have been well characterized [for a review, see MacDonald (1984)], and this technique should offer complementary but novel information about this system.

Materials and Methods

Materials. Gramicidin D (100 mg; Sigma Chemical Co., St. Louis, MO) was dansylated under basic conditions by the method of Veatch and Blout (1976), so as to derivatize only the hydroxyl of Tyr-11 of gramicidin C and not the ethanolamine hydroxyl of gramicidin A, B, or C. Dansyl-gC was isolated from the peptide mixture by chromatography on Sephadex LH-20, on which the dansyl derivative is retarded (Veatch & Blout, 1976). A 4.2-mg aliquot of the resulting dansyl-gC was then further purified by preparative HPLC on phenyl-silica, using the column and method previously described (Koeppe et al., 1985), with the dansyl-gC eluting in 85% methanol. The purity of the final product (Figure 1) was checked was analytical chromatography on a 4.6 × 250 mm column of Zorbax-C8 (DuPont Corp., Wilmington, DE), eluted with 88% methanol at 1.5 mL/min (1800 psi; room temperature). On this column, the dansyl-gC peak had an R_f of 2.3 relative to gramicidin A, whereas gramicidin C has an R_f of 0.7.

Anthroyloxy fatty acids were obtained from Molecular Probes Inc. (Eugene, OR), DMPC was from Avanti Polar Lipids, Inc. (Birmingham, AL), and dansyllysine and dansylalanine were from Sigma Chemical Co. (St. Louis, MO). DMPC was labeled with anthroyloxy probes at a 0.5% molar ratio by cosonication. Dansyl-gC/DMPC small unilamellar vesicles were prepared by cosonication at 1:50 peptide to lipid molar ratios as previously described (Scarlata, 1988). The salt concentration was varied by adding the required amount of sodium or magnesium acetate.

Spectroscopic Measurements. Circular dichroism spectra were taken on a Jasco Mark V in the laboratory of Dr. Esther Breslow. In ethanol no CD spectrum was observed, whereas in bilayers the spectra were dominated by the dansyl residues and showed a large negative band in the near-UV (280–360 nm) and a positive band in the far-UV (220–280 nm). The extinction coefficients at 280 nm for gramicidin and the dansyl amino acids were 22 500 and 20 000 M^{-1} cm^{-1}, respectively, while that of dansyl-gC was found to be 36 500 M^{-1} cm^{-1}.

The fluorescence quantum yield of gramicidin (0.11) in vesicles was taken from Wang et al. (1988), and that of dansyl-gC (0.45) was from Veatch et al. (1975). Fluorescence measurements were performed on an I.S.S. Greg-PC (I.S.S., Champaign, IL). In ethanol, the emission spectrum exciting at 280 nm displayed two peaks, one with a center of spectral mass of 20 kK (500 nm) typical of dansyl in ethanol and another at 28 kK (357 nm) corresponding to tryptophan. In DMPC the centers of mass of these peaks were shifted to 22.5 (444 nm) and 29.7 kK (337 nm), respectively.

Temperatures were varied by a circulating bath. Samples were subjected to hydrostatic pressure in a home-built optical cell based on the design of Paladini and Weber (1981). Lifetime measurements and heterogeneity analyses were performed at in the facility at Mt. Sinai School of Medicine. Polarization and lifetime data were taken with an excitation wavelength of 340 nm and an emission wavelength of 480 nm. Errors in polarization were always ±0.003 or less, while errors in average lifetime were under 50 ps. Changes in energy transfer were monitored by the relative fluorescence intensity at 480 nm when excited at 280 or 340 nm.

Data Analysis. Rotational motion of a fluorophore, determined by the fluorescence polarization (p) or anisotropy (A), is described by the Perrin equation:

$$A_0/A - 1 = (1/p - 1/3)/(1/p_0 - 1/3) = RT\tau/V\eta$$

where A_0 and p_0 are the limiting values, R is the gas constant, T is the absolute temperature, V is the volume assuming spherical rotation, τ is the fluorescence lifetime, and η is the viscosity. The above equation is for isotropic motions of a spherically shaped probe, and this is probably not the case for residues embedded in proteins or membranes. Thus, a direct comparison of the viscosities derived for different residues or the same residue at different locations may not be valid. However, Weber et al. (1984) has shown that the temperature dependence of the viscosity, as expressed in terms of b (see below), was independent of the particular probe or its type of motion and depended only upon the surrounding solvent. This parameter is derived through fluorescence measurements through

$$Y = \ln (A_0/A - 1) - \ln (RT\tau/V) = N + b(T - T_0)$$

where b is termed the thermal coefficient of the viscosity, T_0 is a reference temperature usually chosen to be 273 K, and N is the negative natural log of the viscosity at the reference

temperature. For all solvents, over a certain temperature range (usually ~50 °C), a plot of Y versus the temperature in degrees Celsius ($T - T_0$), termed a Y-plot, yields a straight line from which b is obtained from the slope. b is determined by the change in rotational amplitude of a probe with temperature, and since rotational freedom is determined by the restriction imposed on the fluorophore by the solvent, b is thus related to the thermal expansion of the solvent (Scarlata, 1989). The rationale behind the use of this type of analysis will be later discussed in more detail. The constants used for these calculations have been previously determined (Scarlata, 1989; Weber, 1951): A_0 is 0.312 ± 0.001 for 2-, 6-, and 9-AS, 0.322 ± 0.001 for 11AU, and 0.308 for dansyl, and V is treated as a constant of 150 mL/mol. Average lifetimes ($\sum_i \alpha_i \tau_i$) were used to calculate Y.

In this study we will also analyze our pressure data using an analogous equation to obtain a pressure coefficient of the viscosity (b_p). We have obtained identical b_p values for 2-, 6-, and 9-AS and 11AU (see results) indicating that b_p does not depend on the probe location in the bilayers. The reference pressure (p_0) is taken to be zero.

Results

Energy Transfer. Changes in the amount of energy transfer between tryptophan and dansyl were monitored to determine whether changes in dimer aggregation or conformation occur under various conditions. The efficiency of transfer (E) can be determined by the loss of donor (Trp) intensity through transfer to acceptor, $E = 1 - F/F_0$, where F is the donor intensity in the presence (F) and absence (F_0) of acceptor. The efficiency can also be calculated from the enhancement of dansyl fluorescence that, in the case of transfer from multiple donors, is given by

$$E = F_T/(BQ_a e_D[D])$$

where F_T is the dansyl intensity corrected for direct emission, scattering, and Trp background, Q_a and e_D are the dansyl quantum yield and the tryptophan extinction coefficient, and B is a normalization constant related to the optical conditions of the experiment. Although transfer in two dimensions has been treated, we feel that the above equation is more appropriate here. The parameter B can be determined either by the donor intensity in the absence of acceptor, by the acceptor intensity measured at an excitation wavelength where the donor is not excited, or by comparison to a fluorescent standard. The equations describing this analysis as well as energy-transfer measurements in membrane systems have been recently reviewed (Kleinfeld, 1988).

The efficiency of transfer in DMPC bilayers at 28 °C and atmospheric pressure and 0.1 M sodium acetate was found to be 69 ± 3% by tryptophan quenching and 54 ± 4% by acceptor enhancement where B was determined by either of the three methods described and dansyllysine was used as a control. The larger efficiency determined by donor quenching is characteristic of quantum yield heterogeneity of the tryptophans.

In ethanol, where the CD spectrum was indicative of the intertwined helical form, we find a significant drop in the transfer efficiency to 38 ± 4% by tryptophan quenching and 22 ± 5% by dansyl emission enhancement, showing that this method is sensitive to changes in peptide conformation. This drop is consistent with the tryptophans being more evenly distributed along the helical backbone (Wallace & Ravikumar, 1988; Langs, 1988). For this donor–acceptor pair, Wang et al. (1988) calculated the distance at which 50% transfer (R_0) occurs to be 22 Å. If the orientation factor is assumed to be random, then going from the N to N-terminal dimer to the intertwined dimer decreases the average distance between the dansyl and the Trp's by 6 Å with either the tryptophan quenching or the dansyl enhancement efficiency values. However, while the random orientation factor is a good assumption for the peptide in ethanol, it may not be valid for the peptide in bilayers.

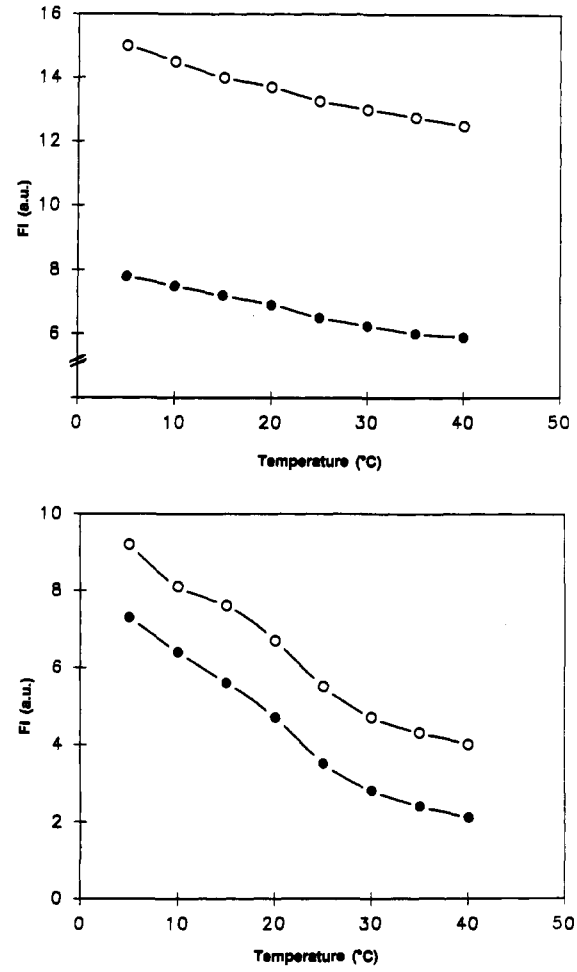

FIGURE 2: (A) Fluorescence intensity (FI) at 480 nm in arbitrary units of dansyl-gC in ethanol exciting at 340 nm (○) and 280 nm (●) as a function of temperature and (B) of dansyl-gC in DMPC bilayers.

To determine whether the peptide conformation or aggregation state was changing with temperature or pressure, we compared the dansyl emission exciting at 280 nm, where the intensity is enhanced from energy transfer, to the untransferred emission using 340 nm excitation. Under these conditions, the changes in the relative intensities at these two exciting wavelengths will directly reflect changes in the transfer efficiency [see Kleinfeld (1988)]. The advantage of comparing two exciting wavelengths to determine variations in transfer efficiency is that it avoids possible artifacts arising from changes in the self-quenching of gramicidin tryptophans, which may decrease with temperature (Scarlata, 1988). Also, the efficiency determined by this method is independent of quantum yield and the number of donors, which may vary with temperature and pressure.

Data were taken from 5 to 40 °C for the peptide in ethanol and in DMPC bilayers. The results in Figure 2 show that both exciting wavelengths produce parallel decreases in intensity. Similarly, no differences in intensity changes were observed upon the application of pressure from 1 atm (~1 bar) to 2 kbar. Thus, no significant changes in the average dansyl-to-tryptophan distance (on the order of a few angstroms) occurs in this temperature and pressure region for either the inter-

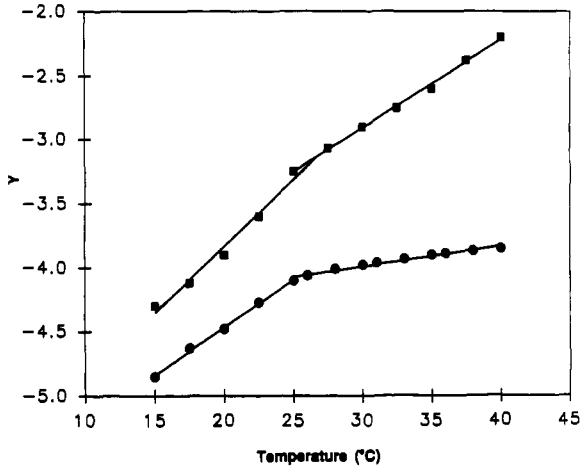

FIGURE 3: Y versus temperature plot of dansyl-gC/DMPC (●) and 11AU/DMPC bilayers (■). An offset of −4.0 was added to the dansyl-gC data to scale the numbers.

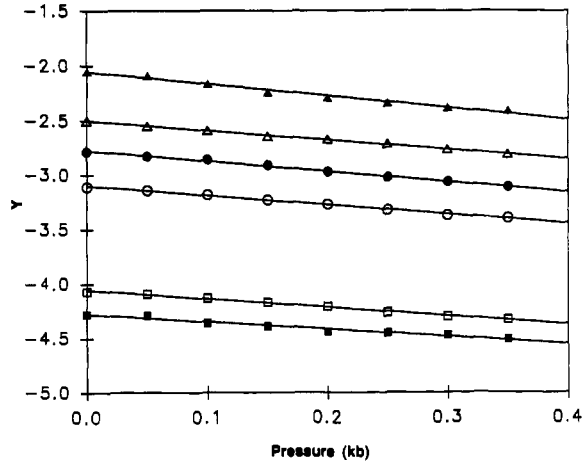

FIGURE 4: Y values as a function of pressure for probes in DMPC small unilamellar vesicles: 11AU (▲), 2AS (△), 9AS (●), 6AS (○), and of dansyl-gC (□) without salt and (■) with 1 M Na^+ at 40 °C. Again, a constant of −4.0 was added to the dansyl-gC data. The higher Y values observed for dansyl-gC without added salt reflect the longer fluorescence lifetimes observed for these samples (see Figure 6).

twined helix in ethanol and or the N to N-terminal helix in DMPC, although we note that in the latter case changes in distance may be compensated for by changes in the orientation factor.

Effects of Fluidity on Dansyl Rotational Motions. Membrane fluidity was initially varied by temperature. The polarization and lifetime of dansyl-gC/DMPC bilayers were observed from 10 to 40 °C. From these data, the change in rotational motion was analyzed in terms of the thermal coefficient of the viscosity or b (see Materials and Methods) and was compared to a fatty acid probe (11AU/DMPC bilayers) under identical conditions. The b values obtained for the anthroyloxy probe represent the increase in rotational amplitude with temperature allowed to the probe by the thermal expansion of the bilayer and is uniform throughout the membrane (Scarlata, 1989). Those for dansyl-gC represent the increase in rotational motion of the probe as allowed by the bilayer and as limited by the attached peptide, which, on the basis of the results of aqueous soluble peptides and proteins, should not expand as rapidly with temperature. Differences in the magnitude of the Y values between the peptide and fatty acid probes are mainly due to differences in anisotropy. In this temperature region both systems show two b values: one corresponding to the gel to liquid-crystalline phase transition (~16–25 °C) and a second corresponding to the fluid or liquid-crystalline phase (25–40 °C). In Figure 3 we see that dansyl-gC is sensitive to the large fluidity changes the membrane undergoes during the phase transition. From these data, we obtain a b value of 0.06 ± 0.01 $°C^{-1}$ for the transition region and 0.015 ± 0.005 $°C^{-1}$ for the fluid phase for which can be compared to 0.12 and 0.072 $°C^{-1}$ values of 11AU/DMPC (Scarlata, 1988).

Membrane fluidity was also varied by the application of high hydrostatic pressure. Since pressure will increase the phase-transition temperature by ~20 °C/kbar [see Heremans (1982)], experiments were done at 40 °C in order to collect data in the fluid phase. The results are shown in Figure 4. The b_p values measured for 11AU, 2AS, 6AS, and 9AS were similar and equal to −0.90 ± 0.05 $kbar^{-1}$. The slopes are negative in this case, representing an increase in the microviscosity with pressure. In the gel phase, nonlinear Y-plots were obtained, and a unique b value could not be obtained. However, in all samples the phase transition occurred at the same pressure. The application of pressure on dansyl-gC/DMPC resulted in a smooth increase in both the fluorescence lifetime and polarization. Unlike the temperature results, the change in dansyl rotational motion with pressure, as determined by b_p, was slightly lower but within error of those obtained for anthroyloxy probes (b_p = −0.80 ± 0.07 $kbar^{-1}$). Thus, change in the rotational motion of dansyl-gC with pressure is similar to the fatty acid probes not attached to a protein.

The change in rotational motion with temperature of dansyl-gC in octyl glucoside or OG micelles was also monitored. Here, the peptide should be in the intertwined helix conformation. The fluorescence polarization and intensity showed a smooth decrease in going from 5 to 40 °C. The Y-plot was linear throughout this temperature range and yielded a b value equal to that of 2AS in OG micelles (0.034 ± 0.002).

Salt Effects. The above experiments were done without added salt. We repeated these measurements in the presence of varying amounts of Na^+, which is permeable, and Mg^{2+}, which is not. Comparisons were made at the same ionic strengths, and in all cases acetate was the counterion. The CD spectrum without salt was identical with those in 1 M Na^+ and 0.33 M Mg^{2+}, indicating that the addition of either salt and the occupancy of the channel by cations does not cause any large conformational changes. While the presence of salt decreased the amount of transfer by ~10%, no significant differences are observed between sodium or magnesium. The temperature behavior of the energy-transfer intensity was unaffected by salt.

The presence of salt did not significantly change the behavior of the polarization with either temperature or pressure (Figure 5). The average lifetimes, however, showed consistently lower values in the presence of Na^+ in the gel phase (Figure 6). To determine whether the observed quenching is a direct effect of sodium, we measured changes in fluorescence intensity of dansylalanine upon NaCl additions in water and found no evidence of quenching, indicating that, in the presence of Na^+, dansyl-gC is subjected to increased solvent quenching. The b values obtained from temperature and pressure experiments were similar in the presence of either salt. Also, the change in rotational motion with temperature of dansyl-gC embedded in octyl glucoside micelles was also unaffected by the addition or the type of added salt.

Heterogeneity lifetime analysis for dansyl-gC in bilayers showed several emitting species that varied slightly with temperature and salt. While the data could be adequately fit to a three-component model (Beechem et al., 1988; Knutson et

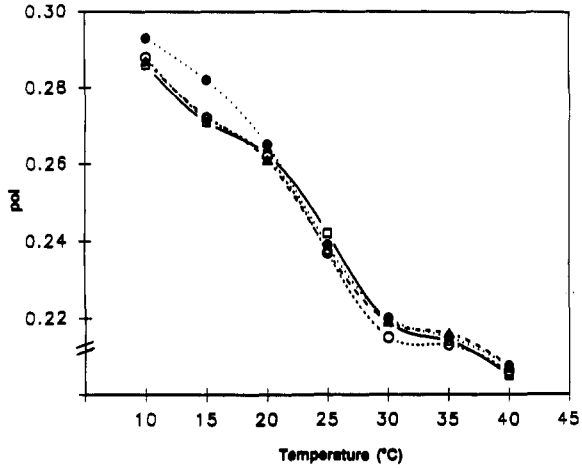

FIGURE 5: Polarization (pol) as a function of temperature of dansyl-gC/DMPC (●) without salt, (□) 0.5 M Na^+, (△) 1 M Na^+, and (○) 0.33 M Mg^{2+}.

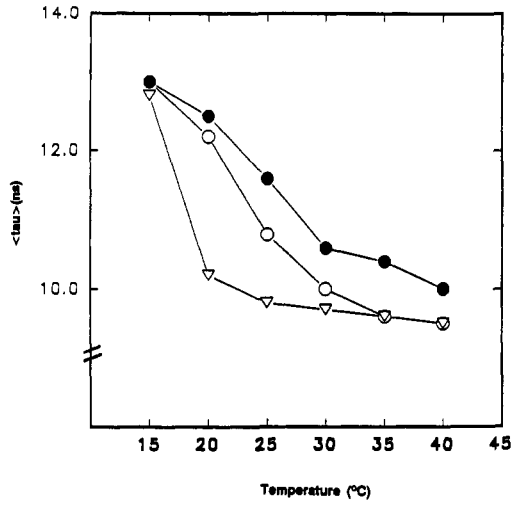

FIGURE 6: Average fluorescence lifetime (τ) versus temperature of dansyl-gC/DMPC (●) without salt, (△) 1 M Na^+, and (○) 0.33 M Mg^{2+}.

al., 1988), the best fits were obtained for four species, two of which were below 2 ns, one at 4–6 ns, and a long-lived component at 13–15 ns. Since the lifetime of dansyl is very short in water, the short-lived population should correspond to those with greater solvent accessibility while the long-lived population corresponds to the most solvent shielded (Royer et al., 1989). Raising the temperature decreases the lifetime of all of the populations. Lifetimes, taken without added salt, in 0.33 M Mg^{2+}, or 1 M Na^+ show similar fractions of species and magnitudes in the fluid phase, but in the gel phase the magnitudes of the two longer lived species were lower in the presence of Na^+ (6.9 and 15.8 ns, as opposed to 7.2 and 16.4 ns in the absence of Na^+ with a maximal error of 0.1 ns), indicating that these populations move into an area of lower dielectric with salt. Attempts to fit the data to models where the lifetime of a particular population changes or the fractional contribution of the population changes with either salt or temperature were unsuccessful.

Discussion

In this study we have characterized the effects of membrane fluidity on the rotational dynamics of dansyl-gC by the parameters derived from the temperature and pressure dependence of the steady-state polarizations and lifetimes. We note that solvent quenching studies of gramicidin D have shown that the peptide stays incorporated in DMPC bilayers at the temperatures and pressures used here (Scarlata, 1991). Our method of analysis, the coefficient of the viscosity (b), has been previously applied to fluorophores in isotropic solvents, peptides, proteins, model membranes, and membrane proteins. In isotropic solvents, Weber et al. (1984) found that b is independent of the rotational properties of the fluorophore and that probes undergoing anisotropic rotations gave the same b value as probes undergoing isotropic motions. For a given solvent, identical b values are obtained either microscopically by fluorescence polarization or macroscopically by flow viscometry. When this analysis was applied to proteins (Scarlata et al., 1984; Rholam et al., 1984), the thermal coefficient of the viscosity always matched the external solvent under highly viscous conditions regardless of the location of the fluorophore in the protein. At lower viscosities, where the fluorophore motions became coupled to neighboring amino acids, lower b values were obtained, indicating a reduced thermal expansion of the local fluorophore environment relative to the solvent. In model membranes where fluorophore motions are asymmetric and the observed polarization can be dissected into static and dynamic components, identical values of b are obtained regardless of the position of the probe in the membrane or whether in-plane or out-of-plane rotational motions are being observed [see the discussion in Scarlata (1989)]. In that study the magnitude of b appears to only depend on the type of lipid comprising the bilayer. This finding can be correlated to recent results of Weber (1989) showing that limited rotations in anisotropic media can be analyzed without geometric restrictions by a thermal equilibrium model.

In this study the rotational motions of the dansyl and the anthroyloxy probes are surely different and asymmetric, leading to polarizations that can only be analyzed by time-resolved methods. However, the *change* in polarization and lifetime with either temperature or pressure will be related to the thermal expansion or isothermal compression of the solvent surrounding the fluorophore. This will be true whether or not exchange of annular lipids occurs during the excited state lifetime. We note that time-resolved measurements of many of these samples yielded inconclusive results due to errors in measurements, whereas steady-state polarization and lifetimes were determined with great accuracy.

From the helical structure of gramicidins, we expect the dansyl residue to be either protruding into the lipid or resting against the peptide surface. In either case, the fluorophore should be exterior enough to be directly affected by changes in membrane fluidity. Similar studies of aqueous soluble proteins show that the motions of exterior residues will be limited by their protein environment, but the viscosity at which these limitations occur is lower than for interior residues (Scarlata et al., 1984; Rholam et al., 1984). The sensitivity of dansyl-gC to changes in membrane fluidity is apparent by the changes in rotational motion with temperature or pressure (Figures 3 and 4). Our results show that the increase in dansyl rotation with temperature, as seen by b, was much lower than that of a fatty acid probe. This indicates that dansyl associates significantly with the peptide, which then limits its increase in rotational motion with temperature. The difference between b values of dansyl-gC/DMPC relative to be b values for fatty acid probes is similar to the difference between aqueous soluble proteins and peptides whose fluorophores are strongly coupled to the amino acids in their subdomain. We thus conclude that dansyl interacts extensively with the peptide. These results imply that the bilayer keeps the peptide sufficiently compact so as to minimize its lipid contacts and the extent of packing

defects caused by the peptide impurity. Our data also show that coupling between the dansyl and the peptide becomes more extensive in the liquid-crystalline phase where the lower viscosity further undamps fluorophore movement and results in increased interactions with the peptide. Increased peptide interactions at lower viscosities are routinely observed in aqueous soluble peptides and proteins (Scarlata et al., 1984; Rholam et al., 1984).

High-pressure results show that the *b* values of dansyl-gC/DMPC are on the same order as that of the fatty acid probes, indicating that the decrease in free volume surrounding the dansyl moiety is similar to that of the lipid. However, the temperature data show that dansyl interacts extensively with the peptide. Since proteins are far more incompressible than lipids (Weber & Drickamer, 1982), we would expect a much smaller change in rotational motion with pressure for dansyl-gC than for 11AU. On the basis of the phase transition, the pressure range explored (0.001–0.35 kbar) has a thermal equivalent of decreasing the temperature from 40 to 25 °C. These data indicate that the much lower change in dansyl rotational motion with temperature as compared to that for the anthroyloxy probes is not due to packing effects but rather results from differences in the mobility of the probes brought about by kinetic energy. While the root of this discrepancy is not clear, we can speculate several possible causes. The dansyl may be located in a primarily peptide environment with a low thermal expansion but with a higher compressibility relative to globular proteins. Aromatic ring stacking as well as changes in the dimensions of the helix would result in a higher compressibility. Also, the application of pressure may force lipid molecules into the space between the dansyl residue and the peptide causing dansyl to be surrounded more completely by the hydrocarbon. We note that since the amount of energy transfer did not change, then any repositioning of the dansyl must not be extensive. Alternately, the free volume around the dansyl residue may be larger than the free volume around the anthroyloxy probes due to packing defects. This larger free volume would compensate for the limitations of the dansyl motions due to the neighboring amino acids. Presently, we cannot distinguish between these mechanisms.

Under our conditions, in DMPC bilayers gramicidin is a formyl-NH to formyl-NH terminal dimer. In micelles, the peptide is an intertwined dimer. In contrast to the DMPC results, the *b* value of dansyl-gC in octyl glucoside micelles matched that obtained for the anthroyloxy probes, indicating that in this conformation the rotational motion of the dansyl is not limited by the peptide. Thus, the micelle must be better able to solubilize the dansyl side chain, keeping it extended into the micelle and not associated with the peptide.

Our results show only subtle differences in the fluorescence parameters of dansyl-gC in the presence and absence of permeable ions: CD shows no changes in secondary structure; energy-transfer measurements shows no significant differences in dansyl–tryptophan distance; polarization measurements shows no differences in the changes in fluidity (which is a function of the effective solvent) with temperature and pressure in either the N–N terminal dimer or the intertwined dimer; and heterogeneity analysis of the fluorescence lifetimes show only small decreases in the presence of Na^+, indicating that dansyl is slightly more solvent oriented. We thus conclude that when the channel is occupied there is no significant reorientation of the aromatic residues. This conclusion is supported by the suggestion of hydrogen bonding between the gramicidin indoles and the lipid head groups (O'Connell et al., 1990; Scarlata, 1991).

Since the dansyl label is so external, the probability of having its rotational motion controlled by solvent should be higher than that of native amino acid residues of integral membrane proteins. Although this was the case in micelles, in bilayers extensive coupling between the probe and the peptide was observed even when the membrane viscosity was very high (i.e., gel-phase DMPC), and at no time do we observe the uncoupling of motions seen in aqueous systems (Scarlata et al., 1984; Rholam et al., 1984). Thus, our data show that membrane viscosity plays a far lesser role in the control of side-chain dynamics than originally thought. However, more proteins must be tested to determine whether these results are general for all membrane proteins.

Acknowledgments

We acknowledge the support of the lifetime facility (NSF Grant DMB-85-16318 to J. B. A. Ross) and thank Drs. J. B. A. Ross, W. Laws, and E. Breslow for the use of and help with their equipment. We also thank Dr. O. Andersen for his helpful comments.

Registry No. gC, 9062-61-7; OG, 41444-50-2; DMPC, 18194-24-6; Na^+, 7440-23-5; Mg^{2+}, 7439-95-4.

References

Andersen, O. S. (1984) *Annu. Rev. Physiol. 46*, 531–548.
Beechem, J., Knutson, J., Ross, J. B. A., Turner, B., & Brand, L. (1988) *Biochemistry 22*, 6054–6058.
Bohg, A., & Ristow, H. J. (1986) *Eur. J. Biochem. 156*, 587–591.
Careri, M., Fasell, P., & Gratton, E. (1979) *Annu. Rev. Biophys. Bioeng. 8*, 69–87.
Cone, R. A. (1972) *Nature 236*, 39–43.
Dannenberg, A. J., Kavecansky, J., Scarlata, S., & Zakim, D. (1990) *Biochemistry 29*, 5961–5967.
Dreyfus, G., Guimaraes-Motta, H., & Silva, J. L. (1988) *Biochemistry 27*, 6704–6710.
Fisher, R., & Blumenthal, T. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 1045–1048.
Gross, E., & Witkop, B. (1965) *Biochemistry 4*, 2495–2501.
Gurd, F., & Rothberg, T. M. (1979) *Adv. Protein Chem. 33*, 73–165.
Heremans, K. (1982) *Annu. Rev. Biophys. Bioeng. 11*, 1–21.
Karplus, M., & McCammon, J. (1981) *CRC Crit. Rev. Biochem. 9*, 293–349.
Kleinfeld, A. (1988) *Spectroscopic Membrane Probes*, Vol. I, pp 63–92, CRC Press, Inc., Boca Raton, FL.
Knutson, J. R., Beechem, J., & Brand, L. (1988) *Chem. Phys. Lett. 102*, 501–507.
Koeppe, R. E., II, Paczkowski, J. A., & Whaley, W. L. (1985) *Biochemistry 24*, 2822–2826.
Langs, D. A. (1988) *Science 241*, 188–191.
MacDonald, A. (1984) *Phil. Trans. R. Soc. London, B 304*, 47–68.
O'Connell, A. M., Koeppe, R. E., II, & Andersen, O. S. (1990) *Science 250*, 1256–1259.
Paladini, A., & Weber, G. (1981) *Rev. Sci. Instrum. 53*, 419–427.
Radda, G. K., & Smith, D. S. (1970) *FEBS Lett. 9*, 287–289.
Rholam, M., Scarlata, S. F., & Weber, G. (1984) *Biochemistry 23*, 6793–6796.
Royer, C., Rusch, R., & Scarlata, S. (1989) *Biochemistry 28*, 6631–6637.
Rudy, B., & Gitler, C. (1972) *Biochim. Biophys. Acta 288*, 231–236.
Scarlata, S. F. (1988) *Biophys. J. 54*, 1149–1157.
Scarlata, S. F. (1989) *Biophys. J. 55*, 1215–1223.

Scarlata, S. F. (1991) *Biophys. J. 59*, 319a.
Scarlata, S., Rholam, M., & Weber, G. (1984) *Biochemistry 23*, 6789-6792.
Shinitzky, M., & Barenholz, Y. (1978) *Biochim. Biophys. Acta 515*, 367-394.
Urry, D. W. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 672-676.
Wallace, B. A. (1990) *Annu. Rev. Biophys. Biophys. Chem. 19*, 127-188.
Wang, S., Martin, E., Cimino, J., Omann, G., & Glaser, M. (1988) *Biochemistry 27*, 2033-2039.
Weber, G. (1989) *J. Phys. Chem. 93*, 6069-6073.
Weber, G., & Drickamer, H. G. (1983) *Q. Rev. Biophys. 10*, 89-112.
Weber, G., Scarlata, S., & Rholam, M. (1984) *Biochemistry 23*, 6785-6789.
Varga, S., Muller, N., Pikula, S., Papp, S., Varga, K., & Martonosi, A. (1986) *J. Biol. Chem. 261*, 13943-13956.
Veatch, W. R., & Blout, E. R. (1976) *Biochemistry 15*, 3026-3030.
Veatch, W. R., Fossel, E. T., & Blout, E. R. (1974) *Biochemistry 13*, 5249-5256.
Veatch, W. R., Mathies, R., Eisenberg, M., & Stryer, L. (1975) *J. Mol. Biol. 99*, 75-92.

Binding and Fluorescence Studies of the Relationship between Neurophysin–Peptide Interaction and Neurophysin Self-Association: An Allosteric System Exhibiting Minimal Cooperativity†

Esther Breslow,*,‡ Thirleen LaBorde,‡ Shakti Bamezai,‡ and Suzanne Scarlata*,§
Departments of Biochemistry and Medicine, Cornell University Medical College, New York, New York 10021
Received February 5, 1991; Revised Manuscript Received April 17, 1991

ABSTRACT: The mechanism of peptide-enhanced neurophysin self-association was investigated to address questions raised by the crystal structure of a neurophysin–dipeptide complex. The dependence on protein concentration of the binding of a broad range of peptides to the principal hormone-binding site confirmed that occupancy of this site alone, and not a site that bridges the monomer–monomer interface, is the trigger for enhanced dimerization. For the binding of most peptides to the principal hormone-binding site on bovine neurophysin I, the affinity of each dimer site was at least 10 times that of monomer under the conditions used. No interactions between the two sites of the dimer were evident. Fluorescence polarization studies of pressure-induced dimer dissociation indicated that the volume change for this reaction was almost 4 times greater in the liganded than in the unliganded state, pointing to a significant alteration of the monomer–monomer interface upon peptide binding. Novel conformational changes in the vicinity of the single neurophysin tyrosine, Tyr-49, induced by pressures lower than required for subunit dissociation, were also observed. The bovine neurophysin I dimer therefore appears to represent an allosteric system in which there is thermodynamic and functional communication between each binding site and the monomer–monomer interface, but no communication across the interface to the binding site of the other subunit. A model for the peptide-enhanced dimerization is proposed in which intersubunit contacts between monomers reduce the large unfavorable free energy associated with binding-induced intrasubunit conformational change. Structural origins of the lack of communication across the interface are suggested on the basis of the low volume change associated with dimerization in the unliganded state and monomer–monomer contacts in the crystal structure. Potential roles for the peptide α-amino group and position 2 phenyl ring in triggering conformational change are discussed.

The binding to neurophysin (NP)[1] of oxytocin, vasopressin, and related small peptides is associated with an increase in the neurophysin dimerization constant, reflecting the stronger binding of peptides to dimer than to monomer (Cohen et al., 1979). This property contrasts with that of such classic allosteric systems as hemoglobin A, in which overall interactions among subunits are weaker in the O_2-saturated tetramer than in the deoxy state (Ackers, 1980), but is potentially related to the O_2-linked strengthening of intersubunit interactions in hemoglobin H (Valdes & Ackers, 1978) and in the final microscopic O_2-binding step of hemoglobin A [see Ackers and Johnson (1990) for a review]. Different mechanisms for the peptide-induced increase in neurophysin dimerization have been proposed (Breslow & Burman, 1990), including the possibility that peptide stabilizes the dimer by binding across the two monomer subunits (Peyton et al., 1986). The demonstration that several peptides produced an identical increase in neurophysin self-association as measured by affinity chromatography was used to argue against the latter mechanism (Fassina & Chaiken, 1988). However, the crystal structure

† Supported by NIH Grants GM-17528 to E.B. and GM-39924 to S.S.
* To whom correspondence should be addressed.
‡ Department of Biochemistry.
§ Department of Medicine. Present address: Department of Physiology and Biophysics, SUNY Stony Brook, Stony Brook, NY 11794-8661.

[1] Abbreviations: NP, neurophysin; Mes, 2-(*N*-morpholino)ethanesulfonic acid; DMF, dimethylformamide; $\bar{p}$, moles of peptide bound per polypeptide chain.

0006-2960/91/0430-7990$02.50/0