Eur Biophys J (1999) 28: 166–173

ARTICLE

Klaus Beyer · Thomas Huber

Mixed micelle formation between gramicidin-S and a nonionic detergent: a nuclear magnetic resonance model study of peptide/detergent aggregation

Received: 22 June 1998 / Revised version: 5 October 1998 / Accepted: 9 October 1998

Abstract The interaction of the cyclic decapeptide antibiotic gramicidin-S (GrS) with the nonionic detergent octaethylene glycol mono-*n*-dodecyl ether was studied by NMR spectroscopy. Detergent binding led to a slightly altered average conformation in the D-Phe side chains of the peptide. The changing diamagnetic shielding of nearby protons resulted in chemical shift variations, the largest effect being observed for the D-Phe C_α proton. The continuous upfield shift of this proton resonance, indicating rapid exchange of the peptide between detergent-associated and unassociated states, was employed for an evaluation of the detergent/peptide aggregation equilibria. The nonlinear binding plot thus obtained was attributed to essentially different aggregational states, depending on the detergent/peptide ratio. The almost linear dependence of the spin-lattice relaxation rate and of the hydrogen-deuterium exchange rate on the fraction of detergent-associated GrS could be reconciled with a simple model, comprising binding of detergent monomers and cooperative binding of micelles at low and high detergent/peptide molar ratios, respectively. Thus, GrS provides a useful model for a study of backbone dynamics and water penetration in detergent- and membrane-bound peptides and proteins. The results will also be discussed with reference to the interaction of GrS with biological membranes.

Key words Cyclopeptide · Surfactant · Interaction · Micelle

Introduction

The structural analysis of membrane proteins by nuclear magnetic resonance (NMR) spectroscopy usually calls for sophisticated solid state techniques and selective isotope labeling (Cross and Opella 1994). As an alternative, well-resolved ^{1}H spectra and negative nuclear Overhauser enhancements can be often obtained in detergent/peptide mixed micelles. It is customary to assume that the detergent micelle provides a good membrane-mimetic environment for the determination of a secondary peptide structure by standard high-resolution methods (Kessler and Seip 1994), neglecting the short average lifetime of globular detergent micelles (Herrmann and Kahlweit 1980). A detergent-bound peptide may be contrasted with a membrane-embedded peptide that is much more effectively shielded from the aqueous environment. Thus, the former usually represents a rapidly exchanging system containing unassociated peptide, mixed peptide/detergent micelles and pure, self-aggregated detergent micelles. Problems associated with micellar exchange, for example, the presence of different aggregational states and of multiple conformers, have not been systematically studied, in spite of the necessity to accumulate a better knowledge of the structure of membrane peptides and proteins (Vinogradova et al. 1997).

In order to explore such equilibria, the cyclic decapeptide gramicidin-S (GrS; *cyclo*-[Val^1-Orn^2-Leu^3-D-Phe^4-Pro^5]$_2$) was chosen as a model that undergoes little conformational change upon binding of the nonionic detergent octaethylene glycol mono-*n*-dodecyl ether ($C_{12}E_8$). GrS forms soluble mixed micellar complexes with the detergent and with phospholipids (T. Huber and K. Beyer, unpublished work), whereas the peptide itself is only sparingly soluble in water. Likewise, it is believed that GrS disrupts microbial cell membranes by the formation of soluble micellar lipid/peptide aggregates (Katsu et al. 1988). Thus, knowledge of the dynamics of these mixed aggregates may also be helpful for an understanding of the antibiotic properties of GrS.

Here we have addressed the problem of the composition and stability of the $C_{12}E_8$/GrS aggregates using high-resolution NMR techniques. The diamagnetic shielding of the D-Phe CH_α ^{1}H resonance was employed for an evaluation of the detergent/peptide aggregation equilibria. Measurements of the spin-lattice relaxation of a geminal

K. Beyer (✉) · T. Huber
Adolf-Butenandt Institut für Physiologische Chemie,
Physikalische Biochemie und Zellbiologie
der Universität München,
München, Germany
e-mail: kbeyer@sparc.pbm.med.uni-muenchen.de

proton pair and of labile hydrogen exchange rates were in line with a simple interpretation in terms of rapid peptide exchange among detergent-associated and unassociated states.

Materials and methods

Chemicals

GrS was purchased from Sigma. The purity of the peptide was checked by thin layer chromatography and proton NMR spectroscopy. Deuterated sodium acetate, deuterium chloride, and sodium deuteroxide were also from Sigma. Octaethylene glycol mono-*n*-dodecyl ether was obtained from Fluka.

Sample preparation

Before weighing, GrS samples were carefully dried. Stock solutions of $C_{12}E_8$ in D_2O or in 80% H_2O + 20% D_2O were added to the preweighed peptide samples to yield the desired detergent/peptide molar ratios. Homogeneous solutions were obtained over the entire range of GrS and $C_{12}E_8$ concentrations applied. For hydrogen exchange experiments, solutions of $C_{12}E_8$ were prepared in D_2O containing 10 mM deuterated sodium acetate. Addition of inorganic buffers for measurements of acidic or basic pH values, e.g. phosphate or borate, resulted in precipitation of the peptide. Thus, the titration was performed in the presence of sodium acetate, even beyond its buffering range. The pD was adjusted by addition of deuterium chloride or sodium deuteroxide using a glass electrode. The pH-meter readings were corrected for the isotope effect according to Glasoe and Long (1960): pD = pH (meter reading) + 0.4. The measurements were repeated after solubilization of the peptide and after the hydrogen exchange measurements. The differences observed were typically < 0.2 pD units. Before mixing, the dry peptide samples and the detergent solutions were equilibrated at 30 °C. The exchange reaction was started by rapid mixing of the components. The decreasing intensities of the individual NH resonances were single exponentials over the entire range from pD = 0.5 to pD = 6.8. The second-order rate constants k_D and k_{OD} were evaluated from the directly determined first-order rates according to Berger et al. (1959):

$$k_{ex} = k_D [D_3O^+] + k_{OD} [OD^-]$$

using a nonlinear least squares procedure. Contributions from buffer ions and water to the exchange catalysis were neglected, as water catalysis plays a minor role, at least close to the pD minimum, and the buffer ions are 10^4 times less concentrated than the solvent water (Englander and Kallenbach 1984). The concentration of the deuteroxide anion, OD^-, was obtained using a dissociation constant $K_{D_2O} = 10^{-14.669}$ at 30 °C, as determined by Covington et al. (1966).

NMR spectroscopy

^{1}H NMR spectra were acquired at 400 MHz using a Varian VXR-400 spectrometer and a 5 mm multi-nuclear probe. Two-dimensional double-quantum filtered shift correlated (COSY) spectra were obtained in the phase sensitive mode according to States et al. (1982). Scalar couplings were extracted from one-dimensional experiments or from the COSY spectra as described in the text. Typically, 2048×512 data points were acquired to obtain sufficient resolution for the determination of individual chemical shifts and coupling constants. Proton exchange rates were obtained at 30 °C by direct observation of the decreasing signal intensity of the labile protons. Sixteen or 32 transients were acquired for each data point and the sampling time was taken as the middle of the corresponding acquisition period. Spin-lattice relaxation times were determined by the standard inversion-recovery method. The rotational state of the D-Phe side chain was derived from vicinal proton-proton coupling constants using the relation $J_{\alpha\beta} = 11.0 \cos^2\theta - 1.4 \cos\theta + 1.6 \sin^2\theta$ (Kopple et al.1973).

Results

Determination of $C_{12}E_8$/GrS binding equilibria by ^{1}H NMR

The formation of detergent/peptide aggregates was monitored using detergent-induced changes in the ^{1}H NMR spectrum of GrS (Fig. 1). Specifically, the α-proton signals of the D-Phe and Pro residues experienced large upfield and downfield shifts, respectively, in contrast to the corresponding signals of the Val, Leu, and Orn residues (Fig. 1 A). At the same time the AB multiplet components arising from the D-Phe C_β protons moved in the opposite direction, passing across each other at a total detergent/peptide molar ratio of 4 : 1 (Fig. 1 B). Owing to the absence of spectral overlap, these signals could be easily identified, even when the detergent was at large molar excess. It may be noted that single spin systems were observed for the five different amino acids during the titration with $C_{12}E_8$, i.e. the time-averaged C_2 symmetry of the cyclic decapeptide persists in the presence of the detergent. Moreover, the absence of separate spectra from detergent-bound and free GrS and the continuous chemical shift changes upon detergent addition indicate that the exchange between different aggregational states is rapid on the NMR timescale.

Titration of GrS with $C_{12}E_8$ results in saturation of the peptide with bound detergent, as shown by the vanishing shift variation at molar ratios >6 (Fig. 2). For a quantitative evaluation of the aggregation equilibria, the detergent-induced shift of the D-Phe CH_α resonance (cf. Fig. 1 A) was analyzed over a wide range of GrS concentrations in the presence of 10 mM $C_{12}E_8$ (Fig. 3). The molar ratio x_b of detergent-associated GrS can be calculated from the observed chemical shift δ, i.e. $x_b = (\delta_f - \delta)/(\delta_b - \delta_f)$. The shift

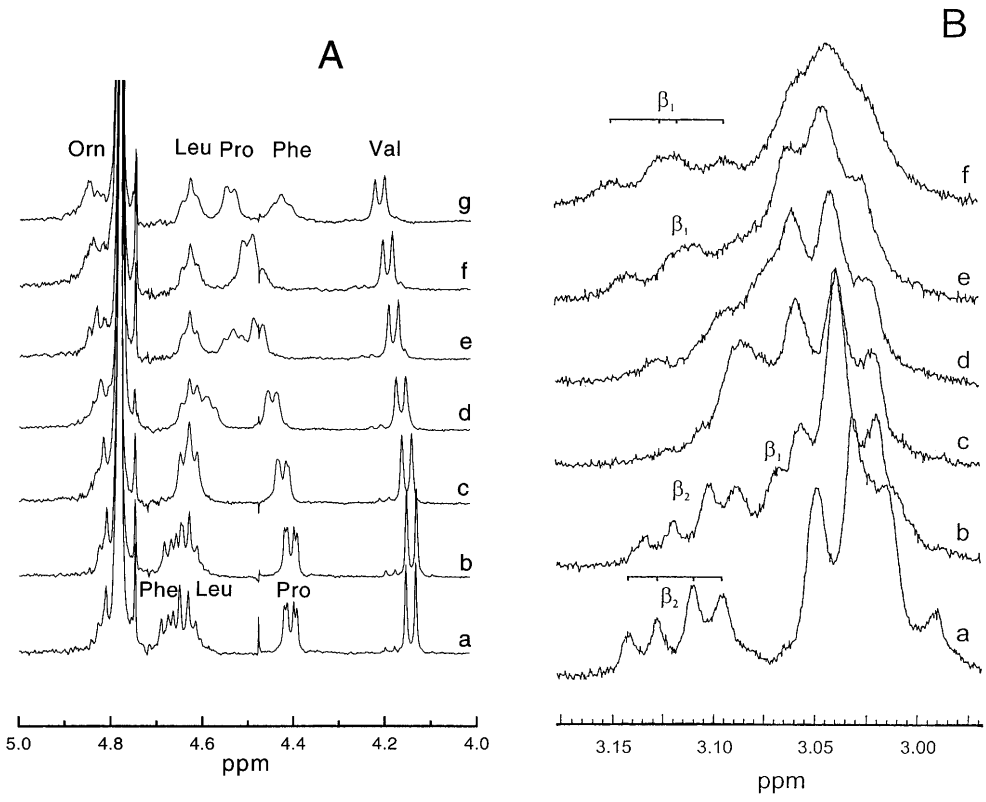


Fig. 1 **A** Detergent-induced changes in the CH_α region of the GrS ^1H NMR spectrum. GrS concentration, 1.85 mM. Molar ratios $C_{12}E_8$/ GrS *a* 0, *b* 5.6×10^{-2}, *c* 1.1×10^{-1}, *d* 5.6×10^{-1}, *e* 1.1, *f* 2.2, *g* 5.6. Temperature, 30 °C. **B** Variation of the AB multiplet arising from the D-Phe C_β proton pair with increasing $C_{12}E_8$ concentrations. Note that the D-Phe CH_β resonances are superimposed by the Orn CH_β resonance at 3.04 ppm. The β_1 and β_2 resonances of the D-Phe residue are assigned in *a* and *f*. Detergent/peptide molar ratios *a* 0, *b* 0.75, *c* 1.49, *d* 4.48, *e* 10.45, *f* 13.44. Total GrS concentration, 1.65 mM

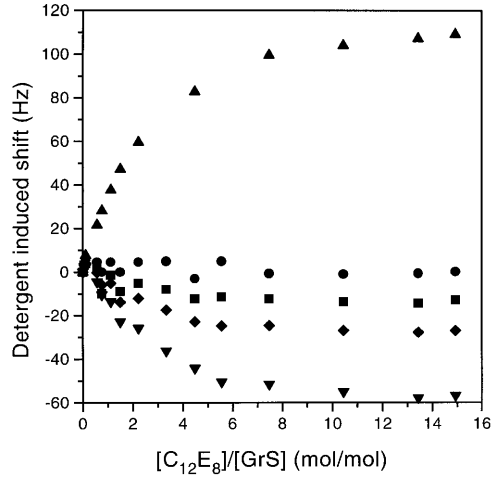


Fig. 2 Detergent-induced shifts of the GrS CH_α signals from the respective signal positions in water alone; D-Phe (▲), Leu (●), Orn (■), Val (◆), and Pro (▼). Total concentration of GrS, 1.85 mM in D_2O

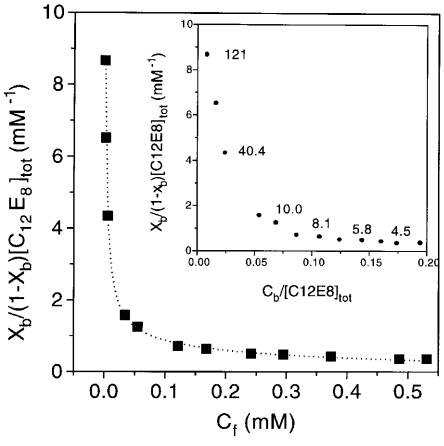


Fig. 3 Representation of the $C_{12}E_8$/GrS aggregation, relating the bound portion of GrS, x_b, with the concentration of unassociated GrS, c_f. The upfield shift of the D-Phe CH_α resonance was used to calculate x_b as described in the text. The *dashed line* is a nonlinear least-squares fit to the data according to Eq. (1). The *inset* shows an alternative representation by analogy to the usual Scatchard plot. The total $C_{12}E_8$/GrS molar ratio is given for selected data points. Total detergent concentration ($[C_{12}E_8]_{tot}$), 10 mM

δ_f in the absence of detergent was taken from a double quantum filtered COSY spectrum (to overcome signal overlap of the Leu and D-Phe α-signals) and δ_b was obtained from an independent titration by extrapolation to zero peptide concentration. The above calculation requires the simplifying assumption of an exchange between two states, i.e. detergent-associated GrS and GrS without detergent binding.

The titration yielded a nonlinear relation between $x_b/(x_b-1)$ and the concentration of unassociated GrS, $c_f=(1-x_b)\,[GrS]_{tot}$ (Fig. 3). Deviation from linearity is to be expected in the context of equilibrium ligand/macromolecule interaction when more than one class of indepen-

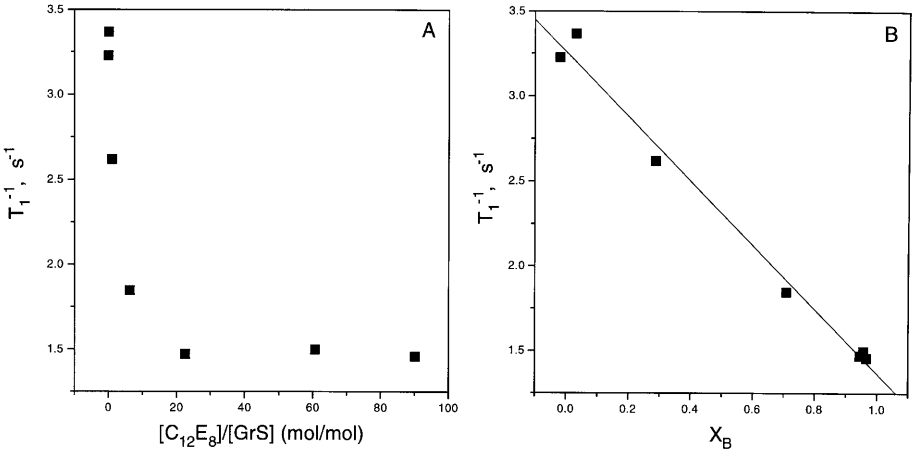


Fig. 4 A, B Dependence of the spin-lattice relaxation rate of the upfield shifted proton of the Pro C_δ proton pair on the detergent/peptide molar ratio (**A**), and on the mol fraction x_b of detergent-associated GrS (**B**). Total GrS concentration, 0.82 mM. Temperature, 30 °C

dent binding sites are involved with different binding constants and different numbers of bound ligands. It is straightforward to analyze the detergent/peptide association analogously, assuming that it can be modeled in terms of ligand binding at two independent sites in a macromolecule (Cantor and Schimmel 1980):

$$x_b/[C_{12}E_8]_{tot}(1-x_b) = n'_1/(K_1+c_f) + n'_2/(K_2+c_f) \quad (1)$$

where $[C_{12}E_8]_{tot}$ denotes the total $C_{12}E_8$ concentration. Equation (1) considers the fraction of detergent-associated peptide that is obtained experimentally (x_b) rather than the fraction of peptide-bound detergent. The constants K_1 and K_2 are microscopic dissociation constants, i.e. $K_m = c_f$ [free site i]/[occupied site i] where i represents a single, independent peptide binding site of class m on the detergent; $m = 1, 2$. Thus, K_1 and K_2 refer to the dissociation of detergent monomers from independent sites in the detergent/peptide complex. It may be noted that Eq. (1) employs the free rather than the bound ligand concentration as an independent variable (in contrast to the conventional Scatchard representation; see insert in Fig. 3). The parameters n'_1 and n'_2 formally embody the "number of binding sites" of GrS on the detergent. Equivalently, $n_1 = (n'_1)^{-1}$ and $n_2 = (n'_2)^{-1}$ are the respective numbers of detergent molecules bound to the peptide in the two different detergent/peptide complexes. A least-squares fit of the four parameters of interest (Bevington 1969) yielded $K_1 = 0.003$ mM and $K_2 = 0.266$ mM for the apparent dissociation constants and $n_1 \approx 32$ mol/mol and $n_2 \approx 5$ mol/mol for the number of bound detergent molecules, respectively.

The constants K_1, n_1 and K_2, n_2 thus obtained correspond with high and low $C_{12}E_8$/GrS molar ratios, respectively (see Fig. 3). It can be concluded that n_1 reflects the composition of the detergent/peptide mixed micelle when the total detergent concentration is not limiting. The size of the mixed micelle may be controlled by the dimension of the peptide, which can be considered as the nucleation center of the aggregate. The number n_2 probably accounts for "first shell" detergent binding at the peptide surface. The large difference between the apparent dissociation constants, i.e. $K_2 \gg K_1$, may then be attributed to the competition between detergent binding to the peptide and detergent self-aggregation (see Discussion).

Rotational motion of the $C_{12}E_8$/GrS complex

The longitudinal relaxation of the geminal proton pair on C_δ of the proline residue can be assumed to reflect the mobility of the GrS backbone structure. This proton pair are well separated in their chemical shifts and the upfield component at 2.61 ppm does not overlap with any other signal even at high detergent concentrations. The relaxation rate $1/T_1$ of the isolated multiplet component decreases with increasing detergent concentration and eventually reaches an almost constant value when $C_{12}E_8$/GrS ≥ 30 mol/mol (Fig. 4 A), indicating slow motion (i.e. $\omega^2 \tau_c^2 \gg 1$, with ω the Larmor frequency and τ_c the motional correlation time) of the corresponding proton-proton vector (Abragam 1961). Thus, assuming rapid exchange (with respect to the spin-lattice relaxation rate, see below) of the GrS molecule between detergent-associated and free states, the relaxation rate is expected to depend linearly on x_b:

$$1/T_1 = 1/T_1^f + x_b[1/(T_1^b + \tau_b) - 1/T_1^f] \quad (2)$$

where T_1^f and T_1^b denote the spin-lattice relaxation rates in the unassociated and detergent-associated states of the peptide, and τ_b the average lifetime of the GrS/detergent complex. An almost linear relationship between $1/T_1$ and x_b was indeed obtained (Fig. 4 B). Equation (2) certainly represents an approximation, as it assumes that T_1^b is invariable over the whole range of detergent/peptide ratios.

The continuous detergent-induced shift of the D-Phe CH_α resonance (> 100 Hz) indicates that $\tau_b \ll 10^{-2}$ s. Therefore $T_1^b \gg \tau_b$ will be a reasonable approximation and $T_1^b \approx 0.73$ s can be obtained by extrapolation to $x_b = 1$. Assuming isotropic tumbling and neglecting relaxation by vicinal and nonbonded protons, a motional correlation time $\tau_c = 1.3 \times 10^{-9}$ s can be calculated from T_1^b for the geminal Pro C_δ proton pair with a distance of 1.8 Å, using standard relaxation theory (Abragam 1961). This correlation time would be compatible with an equivalent sphere having a

radius of 7.6 Å, which may be compared with the long axis of the GrS molecule ($r \approx 12$ Å). The particle dimension thus obtained is almost certainly too small, however, as it neglects local (segmental) oscillations in the β-turn region of the molecule. Thus, it may be assumed that the detergent/GrS stoichiometry of 30 mol/mol corresponds to an aggregate size of 4–5 GrS and 120–150 detergent molecules, the latter value being close to the aggregation number of pure $C_{12}E_8$ micelles (Tanford et al. 1977).

Effect of detergent binding on the H-D exchange rate

Binding of linear amphiphilic peptides to detergent micelles frequently results in structure formation and shielding of the detergent-covered peptide backbone from the surrounding solvent. This is usually demonstrated by measuring the first-order hydrogen-deuterium (H-D) exchange rates of individual backbone amide hydrogen bonds using ^{1}H NMR. There seems to be little or no conformational change in the backbone of the central β-sheet of the GrS molecule upon micelle binding, according to the invariable vicinal J (NH-CH$_\alpha$) couplings (not shown). Thus, it can be assumed that solvent shielding by the detergent rather than stabilization of the secondary structure provides the major contribution to the variation of k_{ex} in the presence of $C_{12}E_8$. An average value of k_{ex} will be obtained if the peptide exchanges rapidly among detergent-associated and unassociated states, as noted above. The rate is expected to depend linearly on x_b, providing that it does not depend on the number of bound detergent molecules (viz. the two aggregation states identified by the Scatchard analysis, cf. Fig. 3), according to

$$k_{ex} = x_b\, k_{ex}^{D} + (1 - x_b)\, k_{ex}^{w} \qquad (3)$$

where the superscripts denote GrS in detergent micelles and in water, respectively.

The dependence of k_{ex} on the $C_{12}E_8$ concentration was obtained by measuring the intensities of Val and Leu amide proton signals at pD 5.4 (Fig. 5). The H-D exchange was too rapid in the case of Phe and Orn, as a result of the different pD minima of these residues. This is shown in Fig. 6, where the computer-simulated curves were obtained by least-squares fitting of the experimental points according to $k_{ex} = k_{OD}\,[OD^-] + k_D\,[D_3O^+]$ with $[OD^-]$ and $[D_3O^+]$ being defined by the respective pD values and by the dissociation constant $K_{D_2O} = 10^{-14.699}$ M^2 (Covington et al. 1966). The chemical shift of the D-Phe CH$_\alpha$ resonance at 4.38 ppm did not change throughout this pH titration, indicating that the bound fraction, x_b, of GrS remains approximately at $x_b = 0.9$.

The linear regression shown in Fig. 5 indicates that the simplifying assumption of a two-state exchange for GrS in the presence of $C_{12}E_8$ micelles is a reasonable approximation. Linear regression of the entire data set for the Val residue gives a k_{ex} value of 2.53×10^{-3} s^{-1} (2.22×10^{-3} s^{-1} as determined without detergent; cf. Fig. 5). The exchange rate without detergent for the Leu residue may be also obtained by extrapolation (15.3×10^{-3} s^{-1}), while it is too fast for direct measurement. The experimental scatter of the data makes an extrapolation of the H-D exchange rates to $x_b = 1$ not feasible.

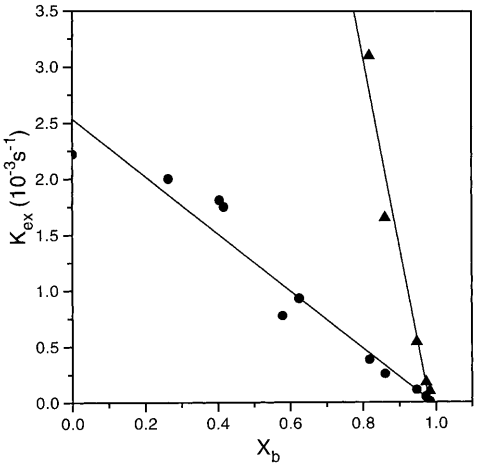


Fig. 5 Dependence of proton-deuteron exchanges rates on the detergent bound GrS mol fraction for Val (●) and Leu (▲). Total GrS concentration, 2 mM. Temperature, 30 °C; pD, 5.4

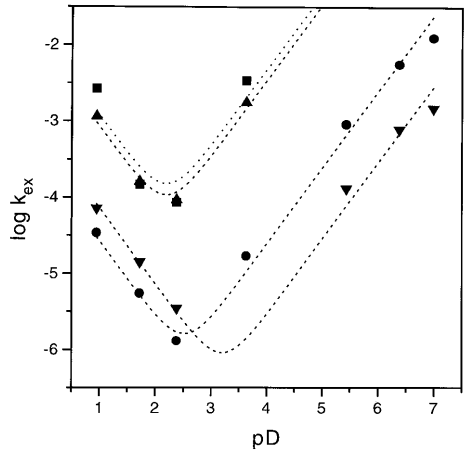
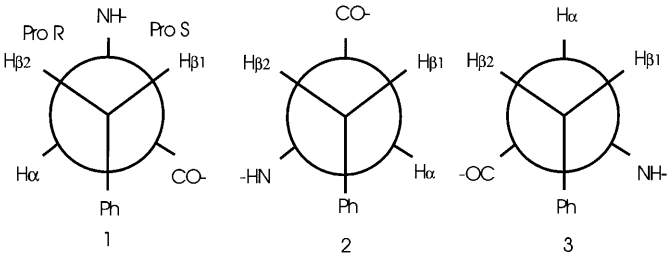


Fig. 6 Proton-deuteron exchange rates k_{ex} of labile NH protons as a function of pD for D-Phe (■), Orn (▲), Leu (●), and Val (▼). The pD values are corrected for the isotope effect. The experimental data were fitted (*dashed lines*) to obtain the second-order rate constants for acid and base catalysis, k_D and k_{OD}, respectively. Total concentration of GrS, 1.65 mM. Ratio $C_{12}E_8$/GrS, 6 mol/mol. Temperature 30 °C

Discussion

Conformation of GrS in $C_{12}E_8$ micelles and biological implications

The secondary structure of GrS represents a small β-sheet, comprising four transannular hydrogen bonds and two β-turns of type II′ (Stern et al. 1968; Ovchinnikov et al. 1970; Ovchinnikov and Ivanov 1975; Rae et al. 1977; Jones



Scheme 1

et al. 1978; Kuo and Gibbons 1979; Kuo et al. 1979; Hawkes et al. 1980; Krauss and Chan 1982a, 1982b; Mirau 1988). The β-sheet structure of GrS persists in a number of different solvents (Ovchinnikov and Ivanov 1975) and in the presence of phospholipid liposomes and lyso-phospholipid micelles (Higashijima et al. 1986).

This also holds true in the detergent mixed micelle according to the invariable backbone scalar couplings and to the reduced proton-deuterium exchange rates. The detergent-induced signal shifts are most probably due to the diamagnetic shielding by the D-Phe ring (Pople 1956). Upon detergent addition the components of the D-Phe C_β proton pair ($CH_{\beta 1}$, $CH_{\beta 2}$) moved in opposite directions (Fig. 1B) and the D-Phe C_α resonance experienced a strong upfield shift. At the same time, there was a slight variation of the vicinal coupling constants $J_{\alpha\beta 1}$ and $J_{\alpha\beta 2}$ of the D-Phe residue from 9.9 Hz to 9.1 Hz and from 6.0 Hz to 7.0 Hz, respectively. The latter observation translates into small changes of the rotamer populations about the D-Phe C_α-C_β bond, i.e. 71%, 29%, and ~0.0% without and 61%, 41%, and ~0.0% with detergent (6/1 mol/mol) for the rotamers 1, 2, and 3, respectively (cf. Scheme 1), while the observed signal shifts are attributable to a slight tilt of the phenyl ring about the C_β-C_γ bond. The dihedral bond angles can be easily reconciled with the invariably large shift difference between the Pro $CH_{\delta 1}$ and Pro $CH_{\delta 2}$ signals, which is also due to the diamagnetic shielding by the nearby aromatic ring (Rae and Scheraga 1978).

The invariable backbone scalar couplings in the detergent micelle are indicative of the stability of the transannular hydrogen bonds that constitute the β-sheet structure of the peptide, a property which may be important for the detergent-like biological function of GrS (Katsu et al. 1987, 1988). The molecular mechanism of the membrane disrupting activity is not clear, although the effect of GrS has been widely studied in cellular and liposomal systems. Most authors agree that the peptide predominantly interacts with membrane phospholipids rather than with non-lipid components of the membrane. In erythrocyte membranes, for example, a parallel increase in lipid release and hemolysis with increasing GrS concentration was demonstrated (Katsu et al. 1989). The prevalence of hydrophobic amino acid side chains on one surface of the molecule suggests that penetration of the membrane-water interface by GrS is a property of these residues, while the positively charged ornithine side chains on the opposite surface remain in contact with the aqueous phase (Highashijima et al. 1986; Katsu et al. 1988). It was concluded that the antibiotic releases massive membrane fragments, which results in membrane defects and membrane permeability (Katsu et al. 1987, 1989). Accordingly, lipid/GrS complexes have been found useful for the transfection of DNA into mammalian cells (Legendre and Szoka 1993). The size and composition of the lipid/GrS complexes were not determined, however, and the details of the GrS conformation in a lipidic environment have not been resolved so far. It seems reasonable to assume that the detergent/GrS aggregates studied here are analogous to the phospholipid/GrS complexes, although the sequence of binding events may be more complicated in biological membranes than in a micellar system.

Detergent/peptide aggregation equilibria

The detergent-induced diamagnetic shielding of the D-Phe CH_α proton was exploited for an evaluation of the equilibria between different aggregational states. A two-step binding process can be recognized from the Scatchard representation in Fig. 3. An aggregate with approximately five detergent molecules per peptide prevails at low overall detergent/peptide ratios, while further addition of detergent eventually leads to a mixed micelle consisting of >30 mol detergent/mol peptide. It may be assumed that the formation of the detergent-saturated mixed micelle is a cooperative process, by analogy with the cooperative binding of the detergents to hydrophobic proteins (Tanford 1980). The size of the detergent/peptide aggregates cannot be deduced from the NMR results alone, however. The almost linear dependence of the proton spin-lattice relaxation rate of the geminal proton pair at Pro C_δ on the micelle-bound fraction of GrS, x_b, suggests that the aggregate size may be nearly constant over the range of detergent additions or, equivalently, that secondary aggregates exist at low overall detergent/peptide molar ratios. The slope would suddenly change upon cooperative detergent binding at high detergent/peptide ratios, in contrast to the almost linear dependence of T_1^{-1} on x_b as shown in Fig. 4.

Three different conditions of aggregate formation may be envisaged, depending on the microscopic dissociation constants for detergent monomers in pure micelles and in the detergent/peptide complex. The molar free energy μ^0_{pep} of detergent monomer binding to the peptide may be substantially more negative than the molar free energy μ^0_{mic} of detergent self-aggregation, i.e. $\mu^0_{pep} \ll \mu^0_{mic}$. Progressive saturation of the high-affinity detergent-binding sites on the peptide surface is then expected to result in an essentially linear Scatchard plot, in contrast to the result shown in Fig. 2. The other extreme, $\mu^0_{pep} \gg \mu^0_{mic}$, implies that pure micelles are preferred and that detergent monomers do not bind to the peptide at all. Finally, competition between detergent/peptide association and detergent self-aggregation ($\mu^0_{mic} \approx \mu^0_{pep}$) will result in a large apparent microscopic dissociation constant for detergent monomer binding in the primary detergent/peptide complex. The microscopic binding constants obtained from the Scatchard plot using

Eq. (1) are in qualitative agreement with the latter conditions, indicating that competition between micelle formation and detergent/peptide association accounts for the biphasic shape of the binding curve in Fig. 3.

Hydrogen exchange kinetics

A sudden decrease of the labile amide hydrogen exchange kinetics upon micelle or bilayer binding of amphiphilic proteins, e. g. phage coat proteins (O'Neil and Sykes 1988; Henry and Sykes 1990) or melittin (Dempsey and Handcock 1996), is usually attributed to the formation of secondary structure. Exchange protection factors $> 10^5$, determined with reference to a completely solvent-accessible random coil polypeptide, poly-D,L-alanine, were obtained for the most slowly exchanging amides in these proteins. Much smaller exchange retardation (5–25-fold) was observed when monomeric secondary amides were incorporated into detergent micelles (O'Neil and Sykes 1989a), indicating that the contribution of solvent shielding by the detergent is small or negligible. Labile hydrogen exchange in the GrS/$C_{12}E_8$ mixtures depends almost linearily on the micellar fraction x_b of the peptide, indicating that the residence time of the peptide in the micellar and in the aqueous phase is short with respect to k_{ex}^{-1} (at least at pH 5.4; cf. Fig. 6). Thus, at moderate detergent concentrations ($x_b < 0.8$) the H-D exchange observed by NMR occurs largely in the aqueous fraction of the peptide. Increasing x_b results in significant exchange protection for the residues that are involved in transannular hydrogen bonding. At the highest detergent/peptide molar ratio that still allowed reliable determination of k_{ex} (38 mol/mol, $x_b = 0.983$), the protection factors relative to the aqueous GrS solution were approximately 150 and 400 for Leu and Val, respectively. Table 1 summarizes the first-order exchange rate constants at the respective pH minima in water (k_{min}^{w}) and in the presence of detergent (k_{min}^{D}) and the pD values where the minima occur (pD_{min}). It can be concluded from the exchange protection in the hydrogen bonded residues Leu and Val that detergent binding stabilizes the antiparallel β-structure of the GrS backbone while the β-turns remain in a mobile, water-accessible state. The values of pD_{min} which corresponds to k_{min} are slightly lower in the presence of the detergent than in water alone, suggesting that in the micellar complex the positively charged side chain of the Orn residue leads to an accumulation of OD^- ions and thereby to a prevalence of base catalysis. This is most significant for the Orn residue itself ($\Delta pD_{min} \approx 0.8$).

Table 1 Hydrogen exchange rate constants and pD-minima

Residue	k_{min}^{w} [a]	k_{min}^{D} [a]	k_{min}^{w}/k_{min}^{D}	pD_{min}^{w} [b]	pD_{min}^{D} [b]
D-Phe	1.1×10^{-3}	1.5×10^{-4}	7.3	2.51	2.20
Val	3.7×10^{-5}	9.0×10^{-7}	41.0	3.24	3.20
Orn	5.4×10^{-4}	1.1×10^{-4}	4.9	2.99	2.20
Leu	7.2×10^{-5}	1.6×10^{-6}	45.0	2.78	2.52

[a] From $k_{min} = 2(k_D\, k_{OD}\, K_{D_2O})^{1/2}$. Values without detergent are from Kraus and Chan (1982b). $C_{12}E_8$/GrS molar ratio 6/1. Concentration of GrS, 2 mg/ml. Note that the rates in water were determined at 21 °C whereas the values with detergent were obtained at 30 °C

[b] From a nonlinear least-squares fit according to $k_{ex} = k_{OD}[OD^-] + k_D[D_3O^+]$

In summary, the present results show that a detergent-solubilized amphipathic peptide exchanges between micelle-associated and unassociated states. Furthermore, detergent binding may induce distinct conformational changes, even in a rigid cyclopeptide. At the same time, the peptide backbone structure is stabilized in the mixed micellar complex. The latter effect of detergent binding may shift the multiple conformational equilibria which are typical for small linear peptides toward the more rigid backbone structures. Thus, obtaining definite structures of amphiphilic peptides in detergent micelles by NMR must be considered with care.

Acknowledgements We thank Brigitte Nuscher for excellent technical assistance. This work was supported by the Deutsche Forschungsgemeinschaft, Sonderforschungsbereich 266, Teilprojekt B12.

References

Abragam A (1961) The principles of nuclear magnetism. Clarendon Press, Oxford

Berger A, Loewenstein A, Meiboom S (1959) Nuclear magnetic resonance study of the protolysis and ionization of *N*-methylacetamide. J Am Chem Soc 81:62

Bevington PE (1969) Data reduction and error analysis for the physical sciences McGraw-Hill, New York

Cantor CR, Schimmel PR (1980) The behavior of biological macromolecules Freeman, San Francisco, p 849

Covington AK, Robinson RA, Bates RG (1966) The ionization constant of deuterium oxide from 5 to 50°. J Phys Chem 70:3820–3824

Cross TA, Opella SJ (1994) Solid-state NMR structural studies of peptides and proteins in membranes. Curr Opin Struct Biol 4: 547–581

Dempsey CE, Handcock LJ (1996) Hydrogen bond stabilities in membrane-reconstituted alamethicin from amide-resolved hydrogen-exchange measurements. Biophys J 70:1777–1788

Englander SW, Kallenbach NR (1984) Hydrogen exchange and structural dynamics of proteins and nucleic acids. Q Rev Biophys 16: 521–655

Glasoe PK, Long FA (1960) Use of glass electrodes to measure acidities in deuterium oxide. J Phys Chem 64:188

Hawkes GE, Randall EW, Hull WE, Convert O (1980) Use of natural-abundance ^{15}N-NMR spectroscopy to investigate the secondary structure of peptides: gramicidin S. Biopolymers 19:1815– 1826

Henry GD, Sykes BD (1990) Hydrogen exchange kinetics in a membrane protein determined by ^{15}N NMR spectroscopy: use of the INEPT experiment to follow individual amides in detergent-solubilized M13 coat protein. Biochemistry 29:6303–6313

Herrmann C-U, Kahlweit M (1980) Kinetics of micellization of Triton X-100 in aqueous solutions. J Phys Chem 84:1536–1540

Higashijima T, Miyazawa T, Kawai , Nagai U (1986) Gramicidin S analogs with a D-Ala, Gly, or L-Ala residue in place of the D-Phe residue: molecular conformations and interactions with phospholipid membrane. Biopolymers 25:2295–2307

Jones CR, Sikakana CT, Hehir S, Kuo MC, Gibbons WA (1978) The quantitation of nuclear Overhauser effect methods for total conformational analysis of peptides in solution. Biophys J 24:815–832

Katsu T, Kobayashi H, Hirota T, Fujita Y, Sato K, Nagai U (1987) Structure-activity relationship of gramicidin S analogues on membrane permeability. Biochim Biophys Acta 899:159–170

Katsu T, Ninomiya C, Kuroko M, Kobayashi H, Hirota T, Fujita Y (1988) Action mechanism of amphipathic peptides gramicidin S and melittin on erythrocyte membrane. Biochim Biophys Acta 939: 57–63

Katsu T, Kuroko M, Morikawa T, Sanchika K, Fujita Y, Yamamura H, Uda M (1989) Mechanism of membrane damage induced by the amphipathic peptides gramicidin S and melittin. Biochim Biophys Acta 983: 135–141

Kessler H, Seip S (1994) NMR of peptides. In: Croasmum WR, Carlson RMK (eds) Two-dimensional NMR spectroscopy. Applications for chemists and biochemists, 2nd edn. VCH, New York Weinheim Cambridge, pp 619–654

Kopple KD, Wiley GR, Tauke R (1973) A dihedral angle-vicinal proton coupling constant correlation for the α-β bond of amino acid residues. Biopolymers 12: 627–636

Krauss EM, Chan SI (1982a) Spectroscopic studies of intramolecular hydrogen-bonding in gramicidin-S. J Am Chem Soc 104: 1824–1830

Krauss EM, Chan SI (1982b) Intramolecular hydrogen bonding in gramicidin S. 2. Ornithine. J Am Chem Soc 104: 6953–6961

Kuo MC, Gibbons WA (1979) Determination of individual side-chain conformations, tertiary conformations, and molecular topography of tyrocidine A from scalar coupling constants and chemical shifts. Biochemistry 18: 5855–5867

Kuo MC, Jones CR, Mahn TH, Miller PR, Nicholls LJF, Gibbons WA (1979) Simplification and spin-spin analysis of the side chain proton magnetic resonance spectrum of the decapeptide gramicidin S using difference scalar decoupling and biosynthesis of specifically deuterated analogs. J Biol Chem 254: 10301–10306

Legendre JY, Szoka FC (1993) Cyclic amphipathic peptide-DNA complexes mediate high-efficiency transfection of adherent mammalian cells. Proc Natl Acad Sci USA 90: 893–897

Mirau PA (1988) Quantitative Interpretation of a single NOESY spectrum. J Magn Reson 80: 439–447

O'Neil JDJ, Sykes BD (1988) Structure and dynamics of a detergent-solubilized membrane protein: measurement of amide hydrogen exchange rates in M13 coat protein by ^{1}H NMR spectroscopy. Biochemistry 27: 2753–2762

O'Neil JDJ, Sykes BD (1989a) NMR studies of the influence of dodecyl sulfate on the amide hydrogen exchange kinetics of a micelle-solubilized hydrophobic tripeptide. Biochemistry 28: 699–707

O'Neil JDJ, Sykes BD (1989b) Side-chain dynamics of a detergent-solubilized membrane protein: measurement of tryptophan and glutamate hydrogen-exchange rates in M13 coat protein by ^{1}H NMR spectroscopy. Biochemistry 28: 6736–6745

Ovchinnikov YA, Ivanov VT (1975) Conformational states and biological activity of cyclic peptides. Tetrahedron 31: 2177–2209

Ovchinnikov YA, Ivanov VT, Bystrov VF, Miroshnikov AT, Shepel EN, Abdullaev ND, Efremov ES, Senyavina LB (1970) The conformation of gramicidin S and its *N,N′*-diacetyl derivative in solutions. Biochem Biophys Res Commun 39: 217–225

Pople JA (1956) Proton magnetic resonance of hydrocarbons. J Chem Phys 24: 1111

Rae ID, Scheraga HA (1978) Shielding effects of the D-Phe aromatic ring in the ^{1}H NMR spectrum of gramicidin S. Biochem Biophys Res Commun 81: 481–485

Rae ID, Stimson ER, Scheraga HA (1977) Nuclear Overhauser effects and the conformation of gramicidin S. Biochem Biophys Res Commun 77: 225–229

States DJ, Haberkorn KA, Ruben DJ (1982) A two-dimensional nuclear Overhauser experiment with pure absorption phase in four quadrants. J Magn Reson 48: 286–292

Stern A, Gibbons WA, Craig LC (1968) A conformational analysis of gramicidin S-A by nuclear magnetic resonance. Biochemistry 61: 734–741

Tanford C (1980) The hydrophobic effect. Wiley, New York

Tanford C, Nozaki Y, Rohde MF (1977) Size and shape of globular micelles formed in aqueous solution by *n*-alkyl polyoxyethylene ethers. J Phys Chem 81: 1555–1560

Vinogradova O, Badola P, Czerski L, Sönnichsen FD, Sanders CR (1997) *Escherichia coli* diacylglycerol kinase: a case study in the application of solution NMR methods to an integral membrane protein. Biophys J 72: 2688–2701