The Conformation of Substance P in Lipid Environments

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ABSTRACT NMR and CD studies have been used to analyze the model membrane-bound structure of the neuropeptide substance P (RPKPQQFFGLM-NH_2, SP), which has previously been proposed as the NK1 receptor active form. Conformations were determined for the SP in the presence of aqueous solutions of zwitterionic dodecylphosphocholine (DPC) and anionic sodium dodecylsulfate (SDS) micelles. The two structures are similar, although fast exchange between free and bound forms was observed for SP with DPC micelles, and predominantly bound characteristics were found for SP in SDS. The addition of 150–200 mM NaCl had no observable effect on the bound conformation in either case. Thus, the structure of SP at a micelle surface is determined largely by hydrophobic forces, and the electrostatic interactions determine the amount of SP that is bound.

INTRODUCTION

Substance P (SP) is a member of the tachykinin family of neuropeptides and is widely distributed in both the central and peripheral nervous system of humans. Tachykinins, which are characterized by a common C-terminal tail (F-*x*-GLM-NH_2, where *x* is F or V for mammals), have been shown to cause hypotension, contraction of smooth muscles, salivation, transmission of pain, and inflammation (Pernow, 1983). SP ligands have potential as a novel class of anti-inflammatory or pain medication, and as a result, there is strong interest in the structural elements of SP that may control receptor activity and selectivity.

Here we report, for the first time, the 3D structure of 1 mM SP when bound to micelles of 60 mM dodecylphosphocholine (DPC). DPC micelles are good models for zwitterionic phospholipid membrane surfaces found in vivo. Findings were obtained via NMR techniques using high lipid-to-peptide ratios. Also determined was the bound 3D structure of SP in the presence of 40 mM sodium dodecylsulfate (SDS) micelles in aqueous solution at pH 5.6, for comparison with SP structure determined previously at a low lipid-to-peptide ratio (Young et al., 1994). The goals of this work were to 1) identify the structural elements of SP that bind to micelles and thus might play a role in receptor binding, and 2) examine the effect of ionic strength and micelle surface charge on peptide association and structure.

SP is selective for the NK1 receptor. Carboxyl fragments of six amino acids or longer retain biological activity (Maggio, 1988), and the N-terminal residues are necessary for receptor subtype selectivity (Ingi et al., 1991). In the NK1 receptor, binding epitopes for the SP C-terminus are located on the receptor C-terminal transmembrane domains V–VII, and the SP N-terminus associates with the NK1 N-terminal transmembrane domain II, the second extracellular loop, and the extracellular N-domain (Yokata et al., 1992).

A structure for SP when bound to a membrane was presented by Schwyzer (1987), who proposed that the nine C-terminal amino acids are inserted as an α-helix perpendicular to an anionic membrane bilayer surface. The mechanism for insertion involved penetration of the C-terminal amino acids into the lipid bilayer followed by diffusion of the peptide in two dimensions to reach the membrane-bound receptor site. Schwyzer also theorized that the membrane surface concentration, conformation, and orientation of a peptide at a lipid bilayer may be more important to the peptide's affinity and specificity for a given receptor than the solution structure (Sargent and Schwyzer, 1986).

Subsequent studies revealed the nature of the lipid surface to be important. Seelig and MacDonald (1989) concluded that SP does not insert into zwitterionic lipid monolayers of 1-pamitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), but it does insert into monolayers with negatively charged 1-palmitoyl-2-oleoyl-sn-glycero-3-phophoglycerol (POPG). A partition coefficient was estimated to be, at most, 41 M^{-1} for 154 mM NaCl, 10 mM Tris, pH 7.4, and "natural" membrane composition (approximately 25% negatively charged lipids). These researchers determine the interaction of SP with negatively charged lipids to be dominated by electrostatic attraction, with peptide hydrophobicity of lesser importance.

Duplaa et al. (1992) report a partition coefficient of 10^6-10^7 for SP in the presence of a negatively charged monolayer of phosphatidylserine in 20 mM sodium phosphate at pH 5.8, whereas circular dichroism (CD) spectra for solutions of SP in the presence of SDS, lysophosphatididylglycerol, and lysophosphatidylcholine micelles indicate an induced helical conformation that is independent of the headgroup present (Woolley and Deber, 1987). Recent NMR studies revealed a partially helical structure for SP in the presence of 15 mM SDS micelles in aqueous solutions at pH 4 (Young et al., 1994).

NMR has been found to be a particularly good method to study micelle-bound peptides. Almost 20 years ago it was

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demonstrated that high-resolution proton NMR spectra can be acquired on peptides bound to micelles of perdeuterated lipids (Brown, 1979), taking advantage of the effective isotropic reorientation of the micelle-bound peptides (McDonnell and Opella, 1993). Conditions for determining the conformations of peptides bound to micelles using NMR have been established by Lauterwein et al. (1979), Braun et al. (1983), McDonnell and Opella (1993), and other researchers. Important is the use of high ratios of lipid-monomer-to-peptide concentrations, typically 20 to 100 times the critical micelle concentration of the lipid. High ratios reduce the possibility of the formation of micelles with more than one peptide present and minimize peptide-peptide interactions. High ratios also ensure a uniform number of lipid monomers per micelle.

MATERIALS AND METHODS

NMR studies

SP was purchased from Sigma Chemical Co. (St. Louis, MO) as the acetate salt and used as delivered. NMR experiments were performed on a Varian Unity Plus 500 MHz spectrometer (Varian Instruments, Palo Alto, CA) with probe air temperature regulated at 25°C. For SP in solution, NMR studies were performed on 1–4 mM peptide in 90% H_2O/10% D_2O; pH was adjusted by HCl. Additional samples were prepared in D_2O to aid in detection of signals near the HOD resonance. pD in these samples was adjusted by the addition of 30% NaOD and 20% DCl in D_2O (CIL, Woburn, MA). pH measurements were performed using an Orion Model 601 pH meter fitted with a combination electrode for 5-mm NMR tubes (Ingold Electrodes, Wilmington, MA). Standard aqueous buffers were used for electrode calibration at pH 4 and 7.

For micelle-bound SP, NMR studies were performed on solutions of 1.0–1.5 mM SP mixed with a) 50–60 mM $CD_3(CD_2)_{11}OPO_3CD_2CD_2N(CD_3)3Cl$ (perdeuterated dodecylphosphocholine or DPC, d_{38} 98.5%; CIL, Andover, MA, and CDN Isotopes, Vaudreuil, Québec) or b) 40–50 mM $CD_3(CD_2)_{11}SO_4Na$ (perdeuterated sodium dodecylsulfate or SDS, d_{25} 98%; CDN Isotopes). For both solutions, pH was adjusted to 5–6 using 1 M NaOH and 1 N HCl. Critical micelle concentrations (CMCs) of DPC and SDS are 1 mM and 8.1 mM, respectively, and the peptide-to-micelle ratios ranged from 1:40 to 1:60 mM.

Chemical shift assignments for SP in solution were obtained by the standard method (Wuthrich, 1986) using the rotating-frame Overhauser effect spectroscopy (ROESY) (Bothner-By et al., 1984), TOCSY (Braunschweiler and Ernst, 1983), and DQFCOSY (Rance et al., 1983) pulse sequences. Chemical shifts for the SP/DPC- and SP/SDS-bound samples were assigned with the standard method using two-dimensional nuclear Overhauser effect spectroscopy (NOESY) (Jeener et al., 1979), total correlation spectroscopy (TOCSY), and double quantum filtered correlation spectroscopy (DQFCOSY) pulse sequences.

For conformational analysis of the SP/lipid mixtures, 100-ms-phase-sensitive NOESY data were used, with 2048 points collected in t_2 (32–64 transients per increment) and 512 complex points in t_1 (States et al., 1982). The transmitter channel was employed for excitation and observation of the 6000-Hz proton frequency range as well as presaturation of the H_2O signal. Additional NOE volumes were obtained using phase-sensitive NOESY data obtained in D_2O. Data were processed with zero filling to 4096 points, with 90° phase-shifted sine-bell apodization functions used in both F1 and F2. 2D NOESY cross-peak volumes were sorted into strong, medium, and weak categories using pro 2 δ proton cross-peak as reference volumes. $^3J_{(NH\text{-}C\alpha H)}$ coupling values were evaluated from resolution-enhanced 1D NMR data by applying unshifted sine-bell apodization to the free induction decays.

CD spectropolarimetry

Circular dichroism (CD) spectra were acquired on samples of 0.8–1.0 mM SP in water and in the presence of 50 mM nondeuterated DPC and SDS (Avanti Polar Lipids, Alabaster, AL) using a Jasco J-600 spectropolarimeter (Jasco, Easton, MD) with a 0.1-mm circular cell. Ten scans were co-added, with data points collected every 0.2 nm at a rate of 20 nm/min from 260 to 185 nm.

Structure calculations: XPLOR

Structures for SP were determined using XPLOR software, Version 3.1 (A. Brunger, Yale University). For SP/DPC, SP/DPC/NaCl, and SP/SDS, the 100-ms and 150-ms NOESY experiments provided the 64, 60, and 70 distance restraints needed for XPLOR 3D structure calculations. Strong, medium, and weak distance restraints were input as 1.8–2.7, 1.8–3.5, and 1.8–5.0 Å distances, respectively, and standard pseudo-atom corrections were used where appropriate (Wuthrich et al., 1983). For SP/DPC, the constraint set contained 32 intraresidue, 21 (i, i + 1), and 11 (i, i + 2, and i + 3) NOEs; for SP/DPC/NaCl, 25 intraresidue, 25 (i, i + 1), and 10 (i, i + 2, and i + 3) NOEs; and for SP/SDS/NaCl, 29 intraresidue, 31 (i, i + 1), and 10 (i, i + 2, and i + 3) NOEs.

Typically, for each distance restraint set, 100 embedded-distance-geometry structures were obtained using dgse protocol. The simulated annealing protocol dgsa was employed to regularize the structures, with a force constant of 50 kcal on the NOE-derived distance restraints and without dihedral and hydrogen bonding constraints (Nigles et al., 1988; Brunger, 1992). The parameter file parallhdg.pro was used in this procedure. Once structures were regularized in this fashion, they were subjected to an additional 10 ps of simulated annealing by the refine protocol. The energy of each regularized and refined structure was then minimized for 200 cycles. Of the resulting 100 structures, 20 were chosen that had no distance violations greater than 0.3 Å and the smallest RMSDs from the mean structure. XPLOR-generated structures were visualized with the program Insight (Version 2.3; Biosym Technologies, San Diego, CA).

RESULTS

NMR chemical shift assignments

^1H chemical shift assignments for SP in aqueous solution and in the presence of DPC and SDS micelles were determined at 500 MHz (Table 1). The assignments obtained in this work for solution-state SP at 25°C and pH 5.4 agree (to within 0.05 ppm) with most of the assignments of Sumner et al. (1990) at 300 MHz, 18°C, and pH 4–6. They also generally agree with the assignments of Young et al. (1994) made at 600 MHz and pH 4, if a correction factor of 0.1 ppm is subtracted from all chemical shifts reported by these researchers. However, chemical shift assignments did not agree with those reported by Chassaing et al. (1986) at 500 MHz, 30°C, and pD 2.5. Disagreement is attributed to differing solution conditions in the two studies.

For SP in the presence of DPC and SDS micelles, most of the amide proton resonances broadened and exhibited large chemical shift changes relative to solution-state SP (Fig. 1). The amide and side-chain proton assignments for SP in the presence of SDS agree with those measured by Young et al. (1994) in 15 mM SDS. However, the chemical shifts of the $C_\alpha H$ protons of residues Q5 to M11 differ by more than 0.05 ppm. Use of a lower micelle-to-peptide ratio (12.5) and pH

TABLE 1 ^{1}H-NMR assignments for substance P in solution* and in the presence of DPC‡ and SDS§ micelles with the chemical shifts relative to H_2O at 4.77 ppm

Residue	NH	$C_\alpha H$	$C_\beta H$	Other		
Arg 1	n.a.	4.18	1.89, 1.80	CγH 1.50	CδH 3.20	NH 7.42
DPC	n.a.	4.31	1.96	1.77	3.24	7.57
SDS	n.a.	4.40	1.92, 1.99	1.78	3.19	7.32
Pro 2		4.46	2.00, 2.30	CγH 1.89, 2.00		CδH 3.55, 3.73
DPC		4.56	1.80, 2.35	2.02		3.54, 3.83
SDS		4.59	1.77, 2.35	1.95, 2.04		3.48, 3.84
Lys 3	8.56	4.52	1.80	CγH 1.50	CδH 1.70	CεH 2.98
DPC	8.54	4.67	1.95	1.57	1.74	3.02
SDS	8.16	4.72	1.92	1.47, 1.52	1.70	3.01
SDS				NH 7.44		
Pro 4		4.36	2.00, 2.28	CγH 1.87, 2.00		CδH 3.61, 3.81
DPC		4.15	1.96, 2.23	1.97, 2.15		3.80
SDS		4.20	2.03, 2.19	1.87, 2.12		3.71, 3.90
Gln 5	8.44	4.18	1.92	CγH 2.27		NδH 6.86, 7.52
DPC	8.78	3.92	1.99	2.37		6.94, 7.72
SDS	8.48	3.80	2.00	2.36		6.79, 7.47
Gln 6	8.26	4.20	1.81	CγH 2.27		NδH 6.86, 7.43
DPC	8.09	4.16	2.00	2.19		6.90, 7.57
SDS	7.94	4.11	2.05	1.93, 2.20		6.80, 7.36
Phe 7	8.18	4.57	3.00, 2.89	2,6H 7.18	3,5H 7.30	4H n.a.
DPC	8.20	4.27	2.95, 3.04	7.05	7.18	n.a.
SDS	7.86	4.35	2.89, 3.00	7.03	7.15	n.a.
Phe 8	8.22	4.57	3.15, 2.92	2,6H 7.24	3,5H 7.34	4H n.a.
DPC	8.02	4.34	3.06, 3.27	7.28	n.a.	n.a.
SDS	7.78	4.39	3.03, 3.26	7.29	7.19	n.a.
Gly 9	7.86	3.78, 3.85				
DPC	8.22	3.87, 3.95				
SDS	8.15	3.90				
Leu 10	8.10	4.33	1.60	CγH 1.60		CδH 0.91, 0.88
DPC	7.84	4.18	1.82	1.57		0.90, 0.94
SDS	7.73	4.20	1.71, 1.81	1.58		0.89, 0.95
Met 11	8.31	4.42	2.00, 2.10	CγH 2.50, 2.60		S-CH_3 2.05
						NH_2 7.08, 7.45
DPC	7.86	4.32	1.99, 2.11	2.41, 2.51		2.00
						NH_2 7.14, 7.30
SDS	7.76	4.31	2.00, 2.11	2.43, 2.51		2.03
						NH_2 6.96, 7.23

n.a., not assigned.
*SP in 90% H_2O/10% D_2O, pH 5.4.
‡SP plus DPC micelles (1:60) in 90% H_2O/10% D_2O, pH 5.4.
§SP plus SDS micelles (1:40) in 90% H_2O/10% D_2O, pH 5.6.

(4.0) by Young et al. than in this work may account for these differences.

Chemical shift difference

Chemical shifts for $C_\alpha H$ and NH signals of SP in solution are plotted versus the random-coil, solution-state chemical shifts of Wuthrich (1986) in Fig. 2 *A*. All chemical shift differences are less than 0.2 ppm, with the exception of G9 and L10, where the amide proton chemical shift differs by 0.5 and 0.3 ppm, respectively. It is noteworthy that Kemmink and co-workers have proposed that the unusual shift for the amide proton of glycine, as seen here, is a general feature of peptides with the sequence Tyr/Phe-X-Gly (Kemmink et al., 1993).

Proton chemical shifts for SP/DPC are plotted against those for SP in solution in Fig. 2 *B*. For SP in the presence of micelles, shifts for the four N-terminal amino acids, RPKP,

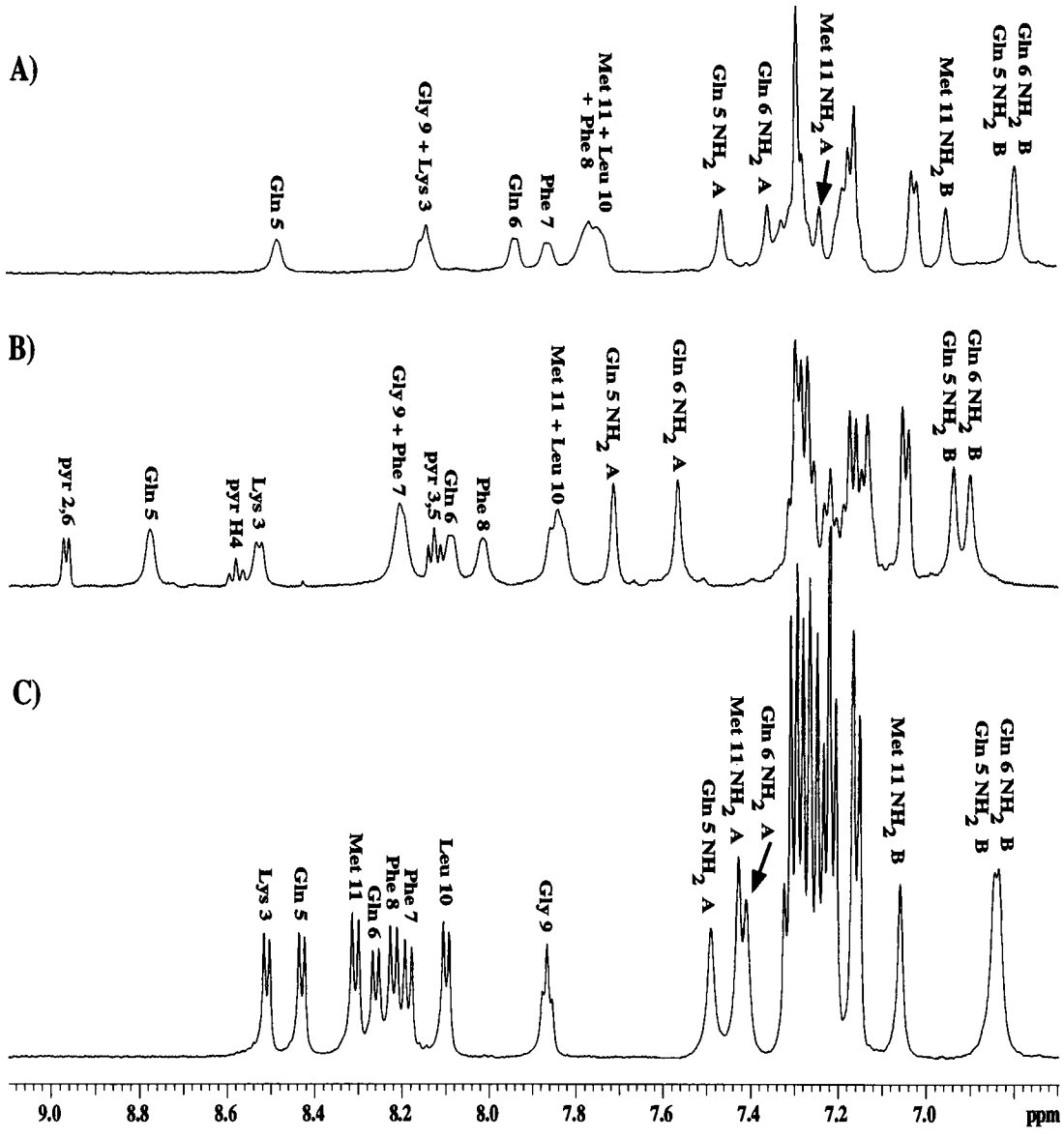

FIGURE 1 The 6.8 to 9.0 ppm region of the 500-MHz ^{1}H-NMR spectra of (*A*) a SP/SDS mixture (1:50) at pH 5.6 in 90% H_2O/10% D_2O, (*B*) a SP/DPC mixture (1:50) at pH 5.3 in 90% H_2O/10% D_2O, and (*C*) SP in 90% H_2O/10% D_2O at pH 6.4. The peaks labeled pyr 2,6, pyr H4 and pyr 3,5 in *B* are signals from the 2,6, 4 and 3,5 protons, respectively, of a pyridine impurity in the DPC.

deviate less than 0.2 ppm from their solution-state values. All C-terminal residues from Q5 to M11, except Q6, differ by more than 0.2 ppm for either the NH or $C_\alpha H$ protons. Chemical shifts for SP/SDS are displayed against those for SP in solution in Fig. 2 *C*. For all residues from Q5 through M11, chemical shifts deviate from random coil values by more than 0.2 ppm for the NH or $C_\alpha H$ protons. Of particular interest is the shift for the SP K3 amide signal, which differs from its solution-state value by 0.4 ppm in the presence of SDS and by only 0.02 ppm in the presence of DPC.

CD spectroscopy

CD spectra from 185 to 260 nm are shown in Fig. 3 for SP in solution, SP mixed with DPC, SP with DPC and NaCl, and SP with SDS. The spectrum for SP in solution is consistent with a random coil structure; the spectra for the SP mixtures are not. For SP/DPC, the spectrum displays a positive peak with a maximum at 195 nm and a minimum at 203 nm, indicating qualitatively that it results from a superposition of SP in random coil and in helical or turn conformations, with the 203-nm minimum representing the isodichroic point. A helical conformation is indicated in the spectrum for SP/SDS, with the maximum at 195 nm and minima at 208 and 222 nm. The CD spectra for SP/DPC and SP/DPC with 150 mM NaCl were identical, thus indicating no change in the ratio of free and bound forms of SP with the addition of NaCl. Similarly, the CD spectra of SP/SDS and SP/SDS with 200 mM NaCl were identical (not shown).

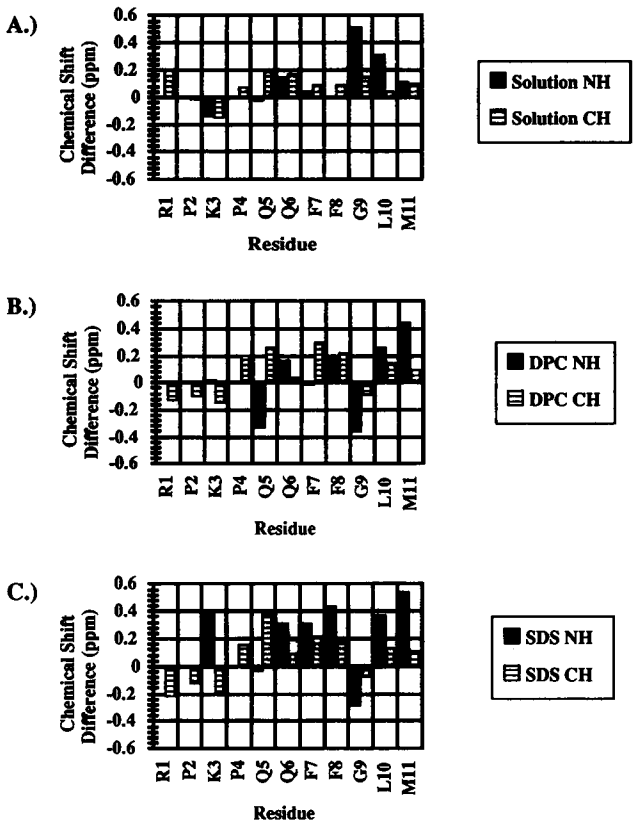

FIGURE 2 Plots of the difference in chemical shift of the NH and $C_\alpha H$ protons of (*A*) SP in 90% H_2O/10% D_2O and pH 5.4 minus the random coil chemical shifts in Wuthrich (1986), (*B*) the SP/DPC mixture chemical shifts minus the SP solution chemical shifts, and (*C*) the SP/SDS solution chemical shifts minus the SP solutions chemical shifts.

From the CD spectra, the mean residue ellipticity at 222 nm was used to estimate the percentage of α-helix present for SP/DPC and SP/SDS, assuming that no other conformations made contributions to the ellipticity at that wavelength (Chen et al., 1974). An α-helical character of 10% was calculated for the SP/DPC solution, based on a value of $-30{,}271$ deg/cm^2 dmol^{-1} for the ellipticity of an 11-residue, 100% helical peptide. Similarly, an α-helical character of 12% was calculated for SP/SDS. In both cases, the small percentage of helical character in the CD spectra could arise from a nonideal helical or turn structure in the peptide at the micelle surface (Williams and Weaver, 1990).

NMR micelle titrations

^{1}H-NMR spectra were collected for SP as a function of the concentration of DPC and, separately, of SDS. pH 5.7 was maintained throughout the titration experiments. As DPC was added to SP, increasing the concentration of DPC micelles, the fast-exchange-averaged chemical shifts of the amide and aromatic protons of SP changed from their solution state values. Steady-state values were reached at DPC:SP ratios of 50:1 or greater. Below this ratio, the concentration of free peptide undergoing fast chemical exchange between free and bound forms was sufficient to effect the observed chemical shifts. At ratios above 50:1 the SP bound proton chemical shifts predominate, although still undergoing fast exchange with a small concentration of free peptide.

For titration of SDS into SP, concentrations of SDS below 8.1 mM (the critical micelle concentration) resulted in formation of SP precipitate; very little SP signal was observed via ^{1}H-NMR. At 11 mM SDS, some of the precipitate cleared and peptide signals appeared in the NMR spectrum; steady-state chemical shifts were obtained at SDS:SP ratios exceeding 20:1. Thus in contrast to SP/DPC solution, SP in the presence of SDS micelles does not undergo fast chemical exchange. Instead, it exists primarily in the bound state, to anionic lipid surfaces.

NMR pH titrations

To investigate further the exchange of SP between free and bound forms in the presence of DPC micelles, ^{1}H-NMR spectra were collected as a function of pH with presaturation of the water resonance. Typically, the exchange rates for unprotected amides over the pH 5 to 7 range result in an increase in the linewidths and a decrease in the intensity of the amide proton NMR signals via saturation exchange with water. If the amides were protected from solvent exchange by secondary structure (e.g., helix, β-sheet, etc. . . .), or by association with the micelle surface, then signals from these protected amide protons would have persisted in the 1D ^{1}H-NMR spectra past pH 7 or higher. ^{1}H-NMR spectra of a SP/DPC mixture collected as a function of pH showed nitrogen-bound proton signals that broadened and disappeared in the 6.6 to 8.0 pH range. In the presence of SDS micelles, the amide proton signals persisted to pHs greater than 9. These observations were consistent with SP undergoing chemical exchange between free and bound forms in the presence of DPC micelles, whereas the peptide was mostly bound to the SDS micelles.

NMR coupling constants

$^3J_{NH\text{-}C\alpha H}$ scalar coupling constants for key amide protons were measured for both SP in solution and the SP/micelle mixtures (Table 2). For SP in solution, coupling constants ranged from 6 to 8 Hz, which is indicative of random coil conformations. Coupling constants for the mixtures varied from this range. For SP/SDS, coupling constants for residues Q5–L10 (excluding G9) fell between 4 and 5.5, which is typical of $^3J_{NH\text{-}C\alpha H}$ values measured for peptide helices (a residue in an ideal α-helix will have a $^3J_{NH\text{-}C\alpha H}$ constant of 3.9 Hz (Wuthrich, 1986)). For SP/DPC, coupling constant values ranged from 4 to 5.5 for residues Q5–L10, except for F7 and Q6 (because SP is undergoing fast chemical exchange between free and bound forms in the presence of DPC micelles, these coupling constants represent the weighted average of the coupling constants for the free

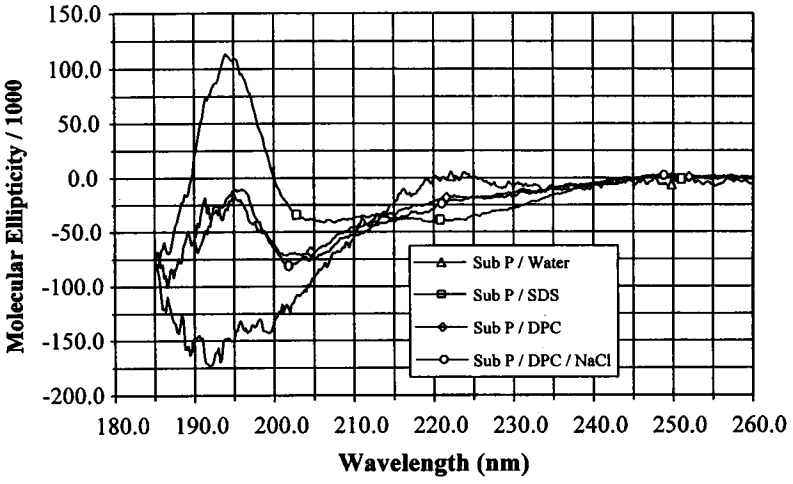

FIGURE 3 A plot of the CD molecular ellipticity from 185 to 260 nm of 0.8 mM SP at pH 5.6 in H_2O (△), in the presence of DPC (1:50) without added NaCl (◇) and with 150 mM added NaCl (○), and in the presence of SDS (1:50) with 150 mM added NaCl (□).

random coil and micelle-bound conformations). For both SP/SDS and SP/DPC mixtures, coupling constant results are consistent with a nonideal helical conformation from Q5–L10, with flexibility at the N- and C-termini.

NMR NOE connectivities

NH-NH and NH-Cα proton correlation regions are shown in Fig. 4, A–D, for the 100-ms NOESY spectra of SP/DPC and SP/SDS. A total of 64, 60, and 70 distance constraints were derived from the NMR data for SP/DPC, SP/DPC/NaCl, and SP/SDS, respectively. For a SP/DPC mixture, the measured NOE volumes increased linearly with mixing time up to 150 ms; at greater mixing times the response was nonlinear. These constraints were used for the calculation of structures satisfying the distance boundaries. Constraints per residue for the Q5–L10 portion of SP were 16, 16, and 18 for SP/DPC, SP/DPC/NaCl, and SP/SDS, respectively, whereas the 2D NOESY spectra for these mixtures showed 11, 10, and 10 interactions between protons on residues separated by two or three residues in the primary sequence of SP (Table 3). Five of the i, $i + 2$, and i, $i + 3$ NOEs in the SP/micelle mixtures were identical, as were seven in the SP/DPC and SP/DPC/NaCl solutions.

XPLOR

Stereo views of the overlay of SP/DPC and SP/SDS XPLOR-derived structures were generated using Insight (Fig. 5, *A* and *B*). SP residues 4 through 10 were superimposed in the stereo views because these residues had the greatest number of distance constraints. Several figures of merit of the overlaid structures are summarized in Table 4. For the most part, the solution structures generated for SP in the presence of DPC micelles with and without added NaCl were identical. This similarity is not surprising, considering that 7 of the 10 i, $i + 2$, and i, $i + 3$ NOEs were the same for both.

For SP/SDS, XPLOR-derived structures also contained many similarities to the structures for SP/DPC. Both overlays show a twisted turn beginning at Q5 that extends through F8, and a second turn over F7 to M11. Furthermore, the N-terminal region of the peptide is very poorly defined by the small number of NOE-derived distance restraints, and a wide range of backbone conformations are possible.

DISCUSSION

SP/DPC

Previous work has shown that the lipid-to-peptide ratio at which the peptide chemical shifts become independent of lipid concentration represents the lower limit for the number of lipid molecules per peptide in the complex (Lauterwein et al., 1979). The ^{1}H-NMR titration results indicated the size of the DPC micelles was about 50 lipid monomers per peptide. Therefore, the molecular weight of a SP/DPC complex would be ~23,000.

TABLE 2 ^{3}J(NH-CαH) coupling constants (in Hz) for SP in solution and in the presence of micelles

Res.	SP Soln.	SP/DPC	SP/DPC/NaCl	SP/SDS	SP/SDS/NaCl
K3	6.3 ± 0.1	7.2 ± 0.4	7.2 ± 0.4	8.2 ± 0.3	6.8 ± 0.3
Q5	6.2 ± 0.1	3.8 ± 0.2	3.6 ± 0.1	3.8 ± 0.3	3.9 ± 0.2
Q6	7.2 ± 0.1	5.7 ± 0.5	6.5 ± 0.5	5.4 ± 0.4	4.9 ± 0.3
F7	7.9 ± 0.2	6.0 ± 0.4	6.3 ± 0.5	5.6 ± 0.6	5.6 ± 0.3
F8	7.7 ± 0.1	4.5 ± 0.5	4.9 ± 0.2	n.m.	4.9 ± 0.4
L10	6.7 ± 0.1	5.3 ± 0.5	5.1 ± 0.4	n.m.	5.1 ± 0.5
M11	7.5 ± 0.2	7.7 ± 0.1	8.3 ± 0.4	n.m.	7.4 ± 0.2

n.m., not measurable. All constants were measured via resolution enhanced 1D data. The reported 1D values are the mean of measurements on three to five spectra.

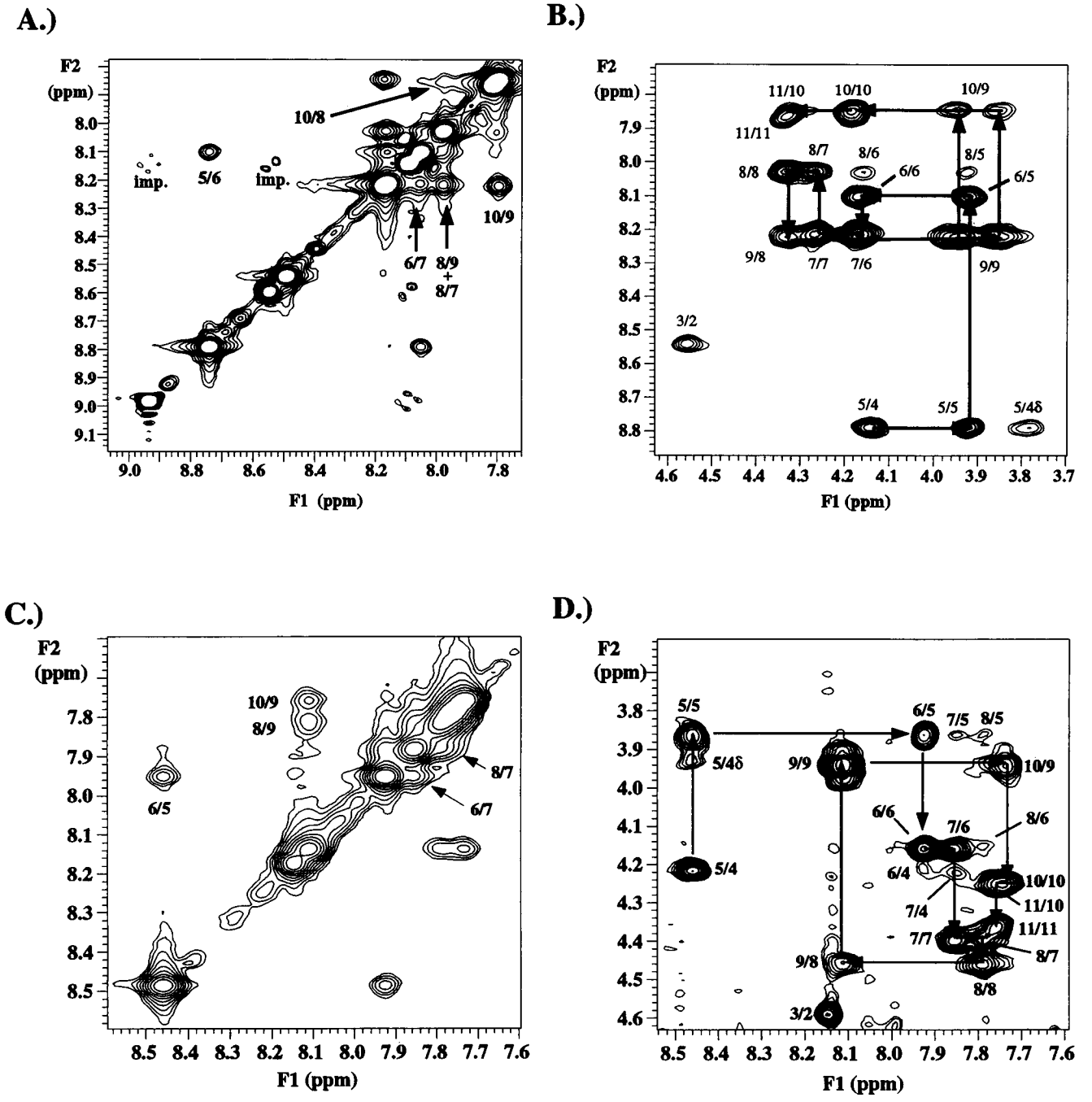

FIGURE 4 Regions of 100-ms NOESYs collected on samples of a SP/DPC mixture (1:60), pH 5.4, with no added NaCl. (*A*) NH to $C_\alpha H$; (*B*) the NH-NH NOEs and a SP/SDS mixture (1:40), pH 5.6, with 200 mM added NaCl; (*C*) NH to $C_\alpha H$; (*D*) the NH-NH NOEs. The cross-peaks labeled imp. in *B* are COSY-type peaks from a pyridine impurity.

Results of NMR and CD studies strongly suggest that SP in the presence of DPC micelles undergoes fast chemical exchange between its random coil solution structure and a micelle-bound conformation. ^{1}H-NMR spectra acquired on a SP/DPC mixture as a function of pH indicated no SP amides that were protected from solvent exchange (protected amides might be expected for stable micelle-bound or stable helical conformations of SP). Thus, the amide exchange occurs via the random coil free SP form that is in chemical exchange with the DPC micelle-bound form.

Qualitatively, the CD spectra of SP/DPC can be interpreted as a mixture of spectral features from SP in a possible helical or β-turn conformation and SP in a random coil conformation. Changes in the solution-state chemical shift

TABLE 3 *i*, *i* + 2 and *i*, *i* + 3 NOEs observed in substance P/micelle mixtures

SP/DPC	SP/DPC/NaCl	SP/SDS/NaCl
Q6 NH K3 CδH	Q6 NH K3 CδH	Q6 NH K3 CδH
F8 NH Q6 CαH	F8 NH Q6 CαH	F8 NH Q6 CαH
F8 NH Q5 CαH	F8 NH Q5 CαH	F8 NH Q5 CαH
F8 CδH L10 CδH	F8 CδH L10 CδH	F8 CδH L10 CδH
F7 CδH L10 CδH	F7 CδH L10 CδH	F7 CδH L10 CδH
L10 NH F8 NH	L10 NH F8 NH	
F7 CαH L10 CδH	F7 CαH L10 CδH	
F7 CδH P4 CαH		F7 CδH P4 CαH
Q5 NH K3 CδH		
	G9 NH F7 CαH	
		Q6 NH P4 CαH
Q5 CαH F8 CβH		
	Q5 CαH F7 CβH	
		F7 NH P4 CαH
F8 CδH Q6 CαH		
	F7 CβH L10 CδH	
		F7 NH Q5 CαH
		F8 CδH L10 CβH

values for SP on titration with DPC are consistent with a greater mole fraction of micelle-bound SP with higher concentrations of DPC. For NMR, the chemical shifts observed represent the average of the chemical shifts of the free and bound forms of SP, which exchange rapidly on the NMR time scale. However, the exchange rate is slow on the CD (or optical spectroscopy) time scale, as indicated by the observed superposition of the spectral features for free and bound forms of SP.

For the 2D NOESY spectrum of SP in solution, NOEs are not measurable because $\omega\tau_c$ is close to unity. Cross-relaxation of proton pairs in the micelle-bound form of SP accounts for the NOEs observed in the NMR experiments. Cross-relaxation rates are much larger for the SP/DPC complex ($\sigma_{(bound)}$) than for solution-state SP ($\sigma_{(free)}$). This follows because under conditions of fast exchange, as is the case for SP/DPC, the average of the cross-relaxation rates will be dominated by $\sigma_{(bound)}$ (as long as a sufficient concentration of bound SP exists). Therefore, the structure calculated from these data is taken to represent the micelle-bound conformation.

The structure for SP bound to DPC micelles that has been determined via NMR analysis is consistent with the weak 10% helicity calculated for this peptide from CD data collected under similar solution conditions. Additionally, the coupling constants (Table 2) measured in the presence and absence of NaCl both show nonideal helicity at residues Q6 and F7 combined with lower coupling constant values in residues Q5, F8, and L10. The twisted-turn structure for SP (Fig. 5) is the first description at the molecular level of this compound in the presence of zwitterionic liquids.

Although increasing the ionic strength of SP/DPC solutions might be expected to alter the fraction of free and bound peptide and thus to alter the exchange-averaged chemical shifts, only small chemical shift changes were observed for the addition of 150 mM NaCl. This suggests a negligible change in the partition coefficient for SP into DPC micelles with the addition of NaCl. Furthermore, the CD spectra show no change on the addition of 150 mM NaCl, and the SP structures determined for the NOE-derived distance restraints show only minor differences. Therefore, the small effect of added salt on the SP/DPC micelle association suggests that SP interacts with zwitterionic micelles predominantly through hydrophobic forces.

Based on the chemical shift differences for DPC micelle-bound SP, as well as the structures determined from NOE data, the C-terminal residues from Q5 to M11 can be identified as those that interact with the micelle surface in a unique conformation, while the RPKP portion remains flexible. Furthermore, the fast exchange that was observed, and the lack of any protected amide signals, suggest that for DPC the peptide C-terminal portion lies parallel to the micelle surface. This conclusion is consistent with a previous observation that SP does not insert into POPC lipid monolayers (Seelig and Macdonald, 1989).

SP/SDS

Previous CD studies, at a pH of 7.3 and 14 mM phosphate buffer, showed changes in SP conformation up to a SDS monomer-to-peptide ratio of 32:1 (Woolley and Deber, 1987). The ^{1}H-NMR data described here showed changes in some of the amide chemical shift values up to a SDS-to-peptide ratio of 17:1. At higher lipid-to-peptide ratios the chemical shifts remained constant. A molecular weight of approximately 30,000 was estimated for the SP/micelle complex by sedimentation velocity analysis (with a SDS-to-peptide ratio of ~100:1) (Holladay and Wilder, 1980; Woolley and Deber, 1987).

Contrasting with the fast exchange that was observed for SP/DPC in NMR and CD studies, SP in the presence of (anionic) SDS micelles appears to be mostly bound; exchange with the free peptide is minimal. This result is consistent with

TABLE 4 Calculated energies and RMSDs (Å) for overlaid structures*

Solution	Constraints	Overlaid	*E*(total) (kcal)	*E*(NOE) (kcal)	RMSD backbone	RMSD heavy atom
SP/DPC	64	20	65.5 ± 6.9	1.5 ± 1.0	0.71 ± 0.14	1.78 ± 0.28
SP/DPC + NaCl	60	20	70.0 ± 7.6	5.1 ± 1.7	0.71 ± 0.11	1.83 ± 0.23
SP/SDS + NaCl	70	15	78.4 ± 4.2	10.2 ± 2.8	0.44 ± 0.12	1.10 ± 0.16

*RMSDs are from the superposition of residues 4–10.

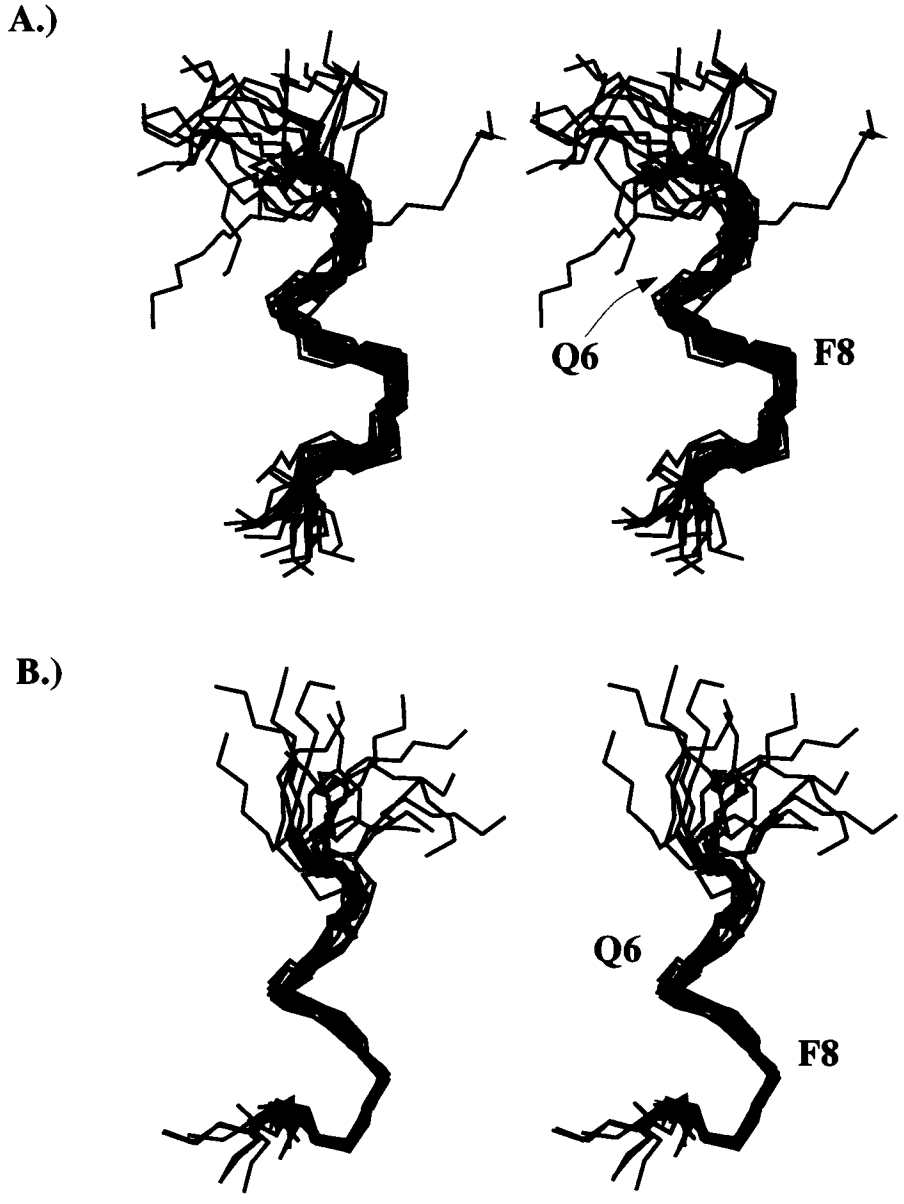

FIGURE 5 Stereo views of the superposition of residues 4–10 of coordinate sets from the XPLOR protocol on SP in the presence of (*A*) DPC micelles with 20 structures overlaid and (*B*) SDS micelles and 200 mM NaCl with 15 structures overlaid.

the previously reported partition coefficients of $\sim 10^6\ M^{-1}$ for anionic SP/1-pamitoyl-2-oleoyl-sn-glycero-3-phosphoserine (Duplaa et al., 1992) and at most 41 M^{-1} for SP/POPC/POPG (Seelig and Macdonald, 1989) mixtures. The structure indicated by CD spectra for SP/SDS indicates weak helicity similar to that of the SP/DPC structure, with a twisted turn from Q5 to F8. Given the 1–2-Å resolution of NMR structure determination methods (Zhao and Jardetzky, 1994), the structures determined for SP in the presence of DPC and SDS micelles are the same.

Increasing the ionic strength of SP/SDS solutions might cause a change in the bound concentration or structure of SP that would lead to changes in the observed NMR chemical shifts and coupling constants. The NMR data showed small changes in the observed chemical shift values and measured scalar coupling constants of SP/SDS mixtures on addition of 200 mM NaCl. In addition, SP/SDS CD spectra showed no change with added salt. Thus, the partition coefficient of SP into anionic SDS micelles is dominated by a large electrostatic component that appears to be unaffected by increasing the ionic strength of SP/SDS solutions.

The findings presented here differ somewhat from those reported by Young et al. (1994). These researchers determined a structure for SP in the presence of 15 mM SDS micelles (lipid-to-peptide ratio 12.5:1) at pH 4, proposing it to represent an equilibrium between an α-helix and a 3_{10}-helix for the midregion of SP. The proposed equilibrium was based on a single dαN i, $i + 4$ and three dαN i, $i + 2$ NOEs from 200 ms NOESY data. Results reported in this study for SP in the presence of 40 mM SDS micelles at pH 5.4 rely on NOESY data at 100 ms and 150 ms (for a SP/DPC mixture, the increase in the NOE volumes was linear with a mixing time up to 150 ms). As a consequence of the shorter mixing times, no dαN i, $i + 4$ NOEs and

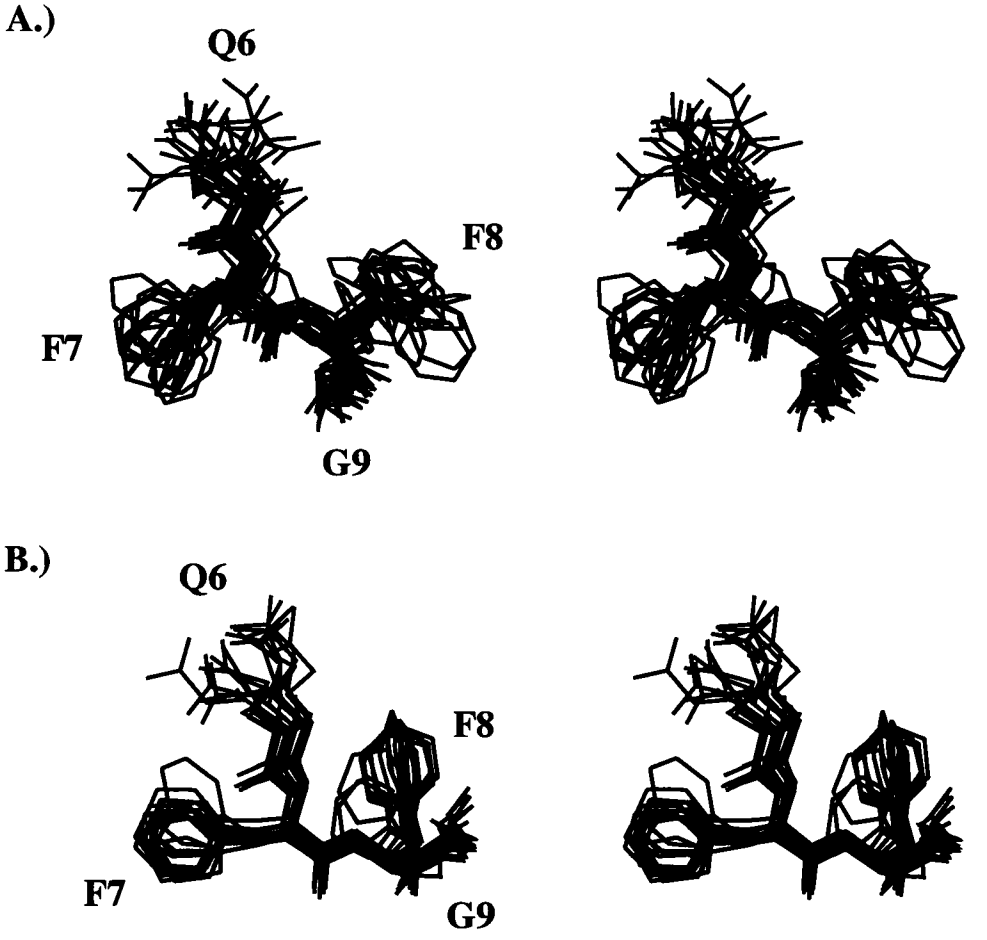

FIGURE 6 Two sets of stereo views of the heavy atom superposition of the QFFG fragment of the superposition of residues 4–10 of (*A*) 20 of the SP/DPC and (*B*) 15 of the SP/SDS coordinate sets generated from the XPLOR protocols.

fewer i, $i + 2$, and i, $i + 3$ NOE connectivities were observed here (10–11 versus 16 for Young et al.); six of these, however, are present at both 15 mM and 40 mM SDS concentrations.

Despite the different chemical shift assignments, NOE connectivities, and calculation methodology of Young et al., the structures determined for SP in the presence of 15 mM SDS are similar to those obtained in this work for SP in 40 mM SDS. Both structures include a single turn about the QFFG part of the peptide and a flexible N-terminal region. In Young et al. the geometry of the turn is reported as approximately α-helical, and the mean backbone ϕ and ψ dihedral angles from P4 to F8 are -70 ± 9 and $-30 \pm 10°$, respectively. Here, the turn in SP is not α-helical and the mean ϕ and ψ dihedral angles from Q5 to F8 are 30 ± 72 and $16 \pm 12°$, respectively.

The variation in turn geometry could result from the difference in the solution conditions employed (15 mM SDS, pH 4 versus 40 mM SDS, pH 5.4, 200 mM NaCl). Chemical shifts for the $C_\alpha H$ protons of Q5 to M11 differ for these conditions, whereas shifts for amide protons are, within experimental error, identical. Thus the structures determined in the two sets of experiments may represent different geometries present under differing solution conditions.

QFFG

In other work, the QFFG fragment of SP has been found to have SP-like activity in an assay using contractile smooth muscle cells from avian amnion (Flood et al., 1994). Based on the structural analyses described above, this fragment exhibits the same relative arrangement of side chains in both SP/DPC and SP/SDS mixtures (stereo view overlays are shown in Fig. 6, *A* and *B*). The arrangement of functional groups in the DPC and SDS micelle-bound form of the SP fragment QFFG could be biologically relevant. The membrane-bound conformation could also play an important role as an intermediate between the solution random coil conformation, and the final receptor-bound form.

A recent model of SP/NK1 receptor interaction suggests that -GLM-NH_2 associates with a transmembrane portion of the NK1 receptor, and that RPKP- interacts with an extracellular region (Li et al., 1995). Interestingly, the most potent NK1 receptor antagonist, CP-99,994, has two aromatic rings that have been suggested to be the recognition elements for the NK1 receptor (Desai et al., 1992). However, for another similar agonist, CP-99,345, the binding site has been shown to be different from the binding epitopes for SP (Gether et al., 1993). Thus the activity for the QFFG fragment and other li-

gands may also be due to binding at NK1 receptor sites not involved in binding SP.

CONCLUSIONS

In previous research on peptides in the presence of lipids, structures have been proposed that are independent of lipid headgroup type for both SP (Woolley and Deber, 1987) and mellitin (Lauterwein et al., 1979). The helical structures proposed by these authors are based primarily on qualitative NMR and CD evidence. This work provides a quantitative basis for the structure of SP in the presence of lipids and its lack of dependence on lipid headgroup type. SP structures were independently calculated to 1–2-Å resolution in three different solution environments: SP/DPC, SP/DPC/NaCl, and SP/SDS/NaCl; all were similar (Fig. 5).

These structures show a unique conformation for association of the QQFFGLM residues of SP with lipid micelles and a variety of conformations for the RPKP- residues at the micelle surface. Similar structural results were found for the conformations of C-terminal residues at an anionic surface, to which SP is strongly associated, and a zwitterionic surface, at which the peptide undergoes chemical exchange that is fast on the NMR time scale and slow on the CD time scale; physiological concentration of NaCl had no observable effect. From these observations we conclude that hydrophobic forces play the primary role in determining the conformation of SP at micelle surfaces, and that electrostatic interaction between the positively charged peptide and the different lipid headgroups determines the amount of time SP spends bound in the lipid environment.

The solution environments employed in these studies (60 mM DPC, 40 mM SDS, pH 5.4, and 150–200 mM NaCl) were selected to minimize peptide-peptide interactions while modeling SP in a charge state like that at physiological pH and NaCl concentrations. Biological membranes, represented in these studies by the micelle surfaces, consist of a mixture of negatively and positively charged lipid with heterogeneous distribution; domains of negatively charged lipids may be induced in large unilaminar vesicles by positively charged peptides (Yang and Glaser, 1995). The interactions of SP with zwitterionic and anionic micelles can serve as a model for the association of SP with biological membrane domains. From the findings of this work, it is thus conceivable that the NK1 receptor could exist in a negative lipid domain that facilities the association of SP. Alternatively, SP might induce a negative lipid domain that plays a role in NK1 activation, similar to the MARCKS peptide activation of protein kinase C (Yang and Glaser, 1995).

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REFERENCES

Bothner-By, A. A., R. L. Stephens, J.-M. Lee, C. D. Warren, and R. W. Jeanloz. 1984. Structure determination of a tetrasacchride: transient nuclear Overhauser effects in the rotating frame. *J. Am. Chem. Soc.* 106:811–813.

Braun, W., G. Wider, K. H. Lee, and K. Wuthrich. 1983. Conformation of glucagon in a lipid-water interphase by ^{1}H-NMR. *J. Mol. Biol.* 169: 921–948.

Braunschweiler, L., and R. R. Ernst. 1983. Coherence transfer by isotropic mixing: application to proton correlation spectroscopy. *J. Magn. Res.* 53:521–528.

Brown, L. 1979. Use of fully deuterated micelles for conformational studies of membrane proteins by high resolution ^{1}H-NMR. *Biochim. Biophys. Acta.* 557:135–148.

Brunger, A. T. 1992. X-PLOR: Version 3.1, A System for X-ray Crystallography and NMR. Yale University Press, New Haven, CT.

Chassaing, G., O. Convert, and S. Lavielle. 1986. Preferential conformation of substance P in solution. *Eur. J. Biochem.* 154:77–85.

Chen, Y.-H., J. T. Yang, and K. H. Chau. 1974. Determination of the helix and beta form of proteins in aqueous solution by circular dichroism. *Biochemistry.* 13:3350–3359.

Desai, M. C., S. L. Lefkowitz, P. F. Thadeio, K. P. Longo, and R. M. Snider. 1992. Discovery of a potent substance P antagonist: recognition of the key molecular determinant. *J. Med. Chem.* 35:4911–4913.

Duplaa, H., O. Convert, A. M. Sautereau, J. F. Tocanne, and G. Chassaing. 1992. Binding of substance P to monolayers and vesicles made of phosphatidylcholine and/or phospatidylserine. *Biochim. Biophys. Acta.* 107:12–22.

Flood, J. F., E. Roberts, M. A. Sherman, B. E. Kaplan, and J. E. Morley. 1994. Topography of a binding site for small amnestic peptides deduced from structure-activity studies: relation to amnestic effect of amyloid β protein. *Proc. Natl. Acad. Sci. USA.* 91:380–384.

Gether, U., Y. Yokota, X. Emonds-Alt, J. Breliere, J. A. Lowe, R. M. Snider, S. Nakanishi, and T. W. Schwartz. 1993. Two nonpeptide tachykinin antagonists act through epitopes on corresponding segments of the NK1 and NK2 receptors. *Proc. Natl. Acad. Sci. USA.* 90: 6194–6198.

Holladay, L. A., and P. Wilder. 1980. Somatostatin-detergent interaction. *Biochim. Biophys. Acta.* 629:156–167.

Ingi, T., Y. Kitajima, Y. Minamitake, and S. Nakanishi. 1991. Characterization of ligand-binding properties and selectivities of three rat tachykinin receptors by transfection and functional expression of their cloned cDNAs in mammalian cells. *J. Pharmacol. Exp. Ther.* 259: 10102–10108.

Jeener, J., B. H. Meier, P. Bachmann, and R. R. Ernst. 1979. Investigation of exchange processes by two-dimensional NMR spectroscopy. *J. Magn. Res.* 71:4546–4553.

Kemmink, J., C. P. M. vanMeirlo, R. M. Scheek, and T. E. Creighton. 1993. Local structure due to an aromatic-amide interaction observed by ^{1}H-nuclear magnetic resonance spectroscopy in peptides related to the N terminus of the bovine pancreatic trypsin inhibitor. *J. Mol. Biol.* 230: 312–322.

Lauterwein, J., C. Bosch, L. R. Brown, and K. Wuthrich. 1979. Physiochemical studies of the protein-lipid interactions in mellittin-containing micelles. *Biochim. Biophys. Acta.* 556:244–264.

Li, Y.-M., M. Marnerakis, E. R. Stimson, and J. E. Maggio. 1995. Mapping peptide-binding domains of the substance P (NK-1) receptor form P388D1 cells with photolabile agonists. *J. Biol. Chem.* 270:1213–1220.

Maggio, J. E. 1988. Tachykinins. *Annu. Rev. Neurosci.* 11:13–28.

McDonnell, P. A., and S. J. Opella. 1993. Effect of detergent concentration on multidimensional solution NMR spectra of membrane proteins in micelles. *J. Magn. Res. Ser. B.* 102:120–125.

Nigles M., G. M. Clore, and A. M. Gronenborn. 1988. Determination of three-dimensional structures of proteins from the interproton distance data by hybrid distance geometry-dynamical simulated annealing calculations. *FEBS Lett.* 2:317–324.

Pernow, B. 1983. Substance P. *Pharmacol. Rev.* 35:85–141.

Rance, M., O. W. Sorensen, G. Bodenhausen, G. Wagner, R. R. Ernst, and K. Wuthrich. 1983. Improved spectral resolution in COSY 1H NMR spectra of proteins via double quantum filtering. *Biochem. Biophys. Res. Commun.* 117:479–485.

Sargent, D. F., and R. Schwyzer. 1986. Membrane lipid phase as catalyst for peptide-receptor interactions. *Proc. Natl. Acad. Sci. USA.* 83: 5774–5778.

Schwyzer, R. 1987. Membrane-assisted molecular mechanism of neurokinin receptor subtype selection. *EMBO J.* 6:2255–2259.

Seelig, A., and P. M. Macdonald. 1989. Binding of a neuropeptide, substance P, to neutral and negatively charged lipids. *Biochemistry.* 28: 2490–2496.

States, D. J., R. A. Haberkorn, and D. J. Ruben. 1982. A two-dimensional nuclear Overhauser experiment with pure absorption phase in four quadrants. *J. Magn. Res.* 48:286.

Sumner, S. C. J., K. S. Gallagher, D. G. Davis, D. G. Covell, R. L. Jernigan, and J. A. Ferretti. 1990. Conformational analysis of the tachykinins in solution: substance P and physalaemin. *J. Biomol. Struct. Dyn.* 8:687–707.

Williams, R. W., and J. L. Weaver. 1990. Secondary structure of substance P bound to liposomes in organic solvents and in solution from Raman and CD spectroscopy. *J. Biol. Chem.* 265:2505–2513.

Woolley, G. A., and C. M. Deber. 1987. Peptides in membranes: lipid-induced secondary structure of substance P. *Biopolymers.* 26:S109–S121.

Wuthrich, K. 1986. NMR of Proteins and Nucleic Acids. John Wiley and Sons, New York.

Wuthrich, K., M. Billeter, and W. Braun. 1983. Pseudo-structures for the 20 common amino acids for use in studies of protein conformations by measurement of intramolecular proton-proton distance constraints with nuclear magnetic resonance. *J. Mol. Biol.* 169:949–961.

Yang, L., and M. Glaser. 1995. Membrane domains containing phosphatidylserine and substrate can be important for activation of protein kinase C. *Biochemistry.* 34:1500–1506.

Yokata, Y., C. Akazawa, H. Ohkubo, and S. Nakanishi. 1992. Delineation of structural domains involved in the subtype specificity of tachykinin receptors through chimeric formation of substance P/substance K receptors. *EMBO J.* 11:3585–3591.

Young, J. K., C. Anklin, and R. P. Hicks. 1994. NMR and molecular modeling investigations of the neuropeptide substance P in the presence of 15 mM sodium dodecyl sulfate micelles. *Biopolymers.* 34:1449–1462.

Zhao, D., and O. Jardetzky. 1994. An assessment of the prescision and accuracy of protein structures determined by NMR; dependence on distance errors. *J. Mol. Biol.* 239:601–607.