Pulsed-Field Gradient and Saturation Transfer Difference NMR Study of Enkephalins in the Ganglioside GM1 Micelle

Chiradip Chatterjee,[†] Barun Majumder,[#] and Chaitali Mukhopadhyay*,[†]

Department of Chemistry, University of Calcutta, 92, A. P. C. Road, Kolkata 700 009, India, and NMR Facility, Department of Biophysics, Bose Institute, P1/12, CIT Scheme 7M, Kolkata 700 054, India

Received: November 21, 2003; In Final Form: February 26, 2004

Alteration of the conformational and dynamic properties of peptides in the presence of micelles mimics the cellular events that take place during insertion and translocation of peptides in biological membranes. We have recently reported the alteration of the conformation of neuropeptides Leu- and Met-enkephalins in the presence of ganglioside GM1 micelles.[41] Here, using pulsed-field gradient-stimulated echo diffusion NMR studies, we observe that the diffusion coefficients of the peptides are greatly reduced in the presence of GM1 micelles. Partition coefficient calculations indicate a nearly total incorporation of the peptides into the micelles. The binding epitope of the enkephalins, as established from the saturation transfer difference NMR spectroscopy, indicates involvement of the Tyr^1, Gly^3, and Phe^4 residues. The involvement of the aromatic side chains of the Tyr^1 and Phe^4 indicates a possible hydrophobic nature of the interaction.

Introduction

The interaction of peptides with biological membranes is central to a number of biological processes, such as the insertion and folding of peptides and proteins in membranes, the rupturing of membranes by toxins, the action of antibiotic peptides, and the membrane-mediated mechanism of peptide−receptor interactions.[1−4] To understand the basis of such interactions, characterization of the altered conformational and dynamic properties of the ligand in the membrane-bound form, mapping of the binding epitope, and so forth, are crucial. A large number of studies using a variety of techniques have been made in recent years on the interaction of peptides with model membranes. Micelles provide a relatively simple membrane system, and they are widely utilized for such studies. For example, in biochemical applications micelles are used to solubilize membrane proteins and as membrane mimics in the conformational analysis of surface-active peptides.[5−15] A large amount of information is also available on peptides binding to SDS and dodecylphosphocholine micelles.[13−18]

NMR-based methods are quite suitable for detecting the binding interactions between small ligands and biomolecular targets.[19] The use of ^{1}H NMR diffusion measurements to investigate solute−micelle binding is a well-established technique. With the help of diffusion NMR measurements one can study the micellar properties and the different hydrodynamic properties of the peptide in the micelle. Hydrophobic, hydrophilic, and electrostatic interactions may all affect the association between peptides and charged micelles. For example, a hydrophilic peptide would be expected either to interact with the charged surface of the micelle or not bind to the micelle at all, while a very hydrophobic peptide would solubilize into the micelle's hydrocarbon core. A peptide may also interact with both the charged surface and the hydrocarbon core if it contains both polar and nonpolar amino acids. The pulsed-field gradient-stimulated echo (PFG-STE)[20] NMR methods have been employed extensively to determine the diffusion coefficient of peptides as well as larger proteins, both in solution and bound to lipid vesicles. These methods have also been used to investigate the aggregation state of biomolecules.[21] Wong and co-workers for example reported the application of the PFG NMR[13−15] experiment to determine the diffusion coefficient of adenocorticotropin peptides in SDS and DPC micelles. The present study utilizes pulsed-field gradient NMR spectroscopy to investigate the interaction between neuropeptide enkephalins and ganglioside GM1 micelles. To obtain information about the binding specificity at an atomic level between the peptide and the micelle and to determine the epitope map of the peptide, we have performed the recently developed STD NMR (saturated transfer difference)[22] experiments.

Compounds with binding affinity to a receptor can directly be identified from a substance mixture, allowing STD NMR to be used as a screening method. It is based on magnetization transfer by receptor signal saturation and its relayed effect to the ligand. During the saturation period, progressive saturation transfers from the receptor to the ligand protons by intermolecular spin diffusion if the ligand binds to the receptor. The signals of the ligands are made visible by subtracting the saturated spectrum from one without receptor saturation. Additionally, the ligand's binding epitope can also be determined from the spectra. The ligand protons nearest to the receptor should be saturated to the highest degree and, therefore, have the strongest signal in the STD spectrum. The ligand protons, which are further from the target surface, will be saturated to a lower degree, and their STD intensities will be weaker. Therefore, the degree of saturation of the individual ligand protons reflects their proximities to the receptor surface and can be used as an epitope method to describe the target ligand interactions.[22−30]

We have studied the effect of ganglioside GM1 micelles on neuropeptide enkephalins. Enkephalins are endogenous neuropeptides having opioid-like activity and compete with mor-

* Corresponding author. Fax: 91-033-2351-9575. E-mail: chaitalicu@yahoo.com; chaitali@cucc.ernet.in.
[†] University of Calcutta.
[#] Bose Institute.

10.1021/jp037553r CCC: $27.50 © 2004 American Chemical Society
Published on Web 05/07/2004

phine for the receptor binding.[31,32] The binding of enkephalin neuropeptides to the membrane appears to be crucial for their action since they interact with the nerve cell membrane to achieve bioactive conformations that will then fit onto the multiple receptors (μ, δ, and κ) [21,33−35]. It is thus important to study the conformation of enkephalins in different membrane mimetic conditions. We have chosen ganglioside GM1 as the micelle-forming lipid. Gangliosides are sialic acid containing glycosphingolipids that are ubiquitous components of mammalian plasma membranes[36] and constitute a significant fraction of the brain lipids.[37] They are found in relative abundance in nervous tissue where the cell-surface location and the temporal and spatial regulation of the expression of specific ganglioside species[38,39] have prompted investigation into the roles that these molecules play in nervous system development and function. It has been observed that enkephalins affect the synthesis of gangliosides in several cell lines.[40]

In our previous paper,[41] we reported the solution conformations of enkephalins (LE and ME) in the presence and absence of GM1. It was observed that both the enkephalins interact with GM1, and there is a distinct change in the conformations of these two opioid peptides upon binding to GM1. The present study gives detailed information about the change in hydrodynamic properties and identifies the binding epitope of enkephalins with the GM1 micelle. With the use of the pulsed-field gradient-stimulated echo (PFG-STE) diffusion NMR and saturation transfer difference NMR experiments, the direct interaction and the partitioning of enkephalins in the ganglioside GM1 micelle are reported here.

Materials and Methods

Leu-enkephalin and Met-enkephalin were purchased from Sigma Chemical Co. (St. Louis, MO) with 97% purity and used without further purification. Neuraminidase enzyme (Type VI) was purchased from Sigma Chemical Co. (St. Louis, MO). DEAE-Sephadex was obtained from Pharmacia (USA). IR-120 cation exchange resin was from Mallinckrodt Chemical Works (New York). All the isotopically enriched ^2H_2O was obtained from ISOTEC Inc. (USA). Water used for NMR was of Millipore grade.

GM1 was isolated and purified from goat brain in our laboratory following the published protocol.[42,43] After dressing, that is, washing and removing the blood vessels, the brain tissue was homogenized with a chloroform/methanol (2:1 v/v) mixture in a blender. After extraction it was treated with 0.2% KCl solution and kept overnight for phase separation. Gangliosides were obtained from the upper aqueous phase. The upper phase was collected, diluted with methanol to reduce the salt concentration below 0.04M, and passed through a DEAE-Sephadex column (15 cm × 2 cm). The column was washed thoroughly with methanol and eluted with 0.4 M ammonium acetate. The eluted mixture was dialyzed extensively and lyophilized. The ganglioside GM1 was finally prepared from the mixture of gangliosides by neuraminidase digestion[44] which removes sialic acid from polysialogangliosides but does not further degrade the monosialoganglioside GM1. The total ganglioside from goat brain was incubated at 37 °C for 30 min with neuraminidase enzyme to obtain the monosialo variety of ganglioside or GM1. After lyophilization GM1 was dissolved in water and passed through a cation exchange IR-120 resin column to remove any cations present. After extensive dialysis and lyophilization, white powdered GM1 was obtained whose purity was checked by TLC and ^{1}H NMR spectroscopy.[45,46] With repeated TLC experiments, we consistently obtained only one single spot which indicates the presence of GM1 only in the purified lipid. To make sure, we also measured the sugar/sialic acid ratio using a standard protocol[47] and found a confirmative 3:1 ratio.

All NMR experiments were performed on a Bruker DRX 500-MHz spectrometer equipped with a 5-mm broadband inverse probe head at 300 K. The maximum gradient strength was calibrated by the known diffusion coefficients of water.[48] For diffusion experiments, the longitudinal eddy current delay (LED) pulse sequence was used.[49] The diffusion time Δ was 300 ms, and the duration of the gradient pulse, δ, was 5 ms. The relaxation delay was 30 s. A total of 32 scans were obtained for each spectrum. PFG was applied in both the *x*- and *y*-directions for micellar samples and only in the *x*-directions for aqueous samples. The gradient strength in a series of experiments was typically incremented from 7 G/cm to ∼50 G/cm in 10 steps. Peak integrals were collected at each gradient strength, and the intensities (I) of the integrals were related to the diffusion coefficient (D) by[50]

$$I = I_0 \exp\left[-DG^2\gamma^2\delta^2(\Delta - \delta/3)\right] \quad (1)$$

where I_o is the signal intensity in the absence of a gradient pulse, Δ is the diffusion delay time, δ is the length of the gradient pulse, γ is the gyromagnetic ratio of the nucleus, and G is the gradient pulse amplitude.

For fast exchange, the mole fraction of bound peptide, f_b, was related to the observed diffusion coefficient, D_{obs}, according to[18,51]

$$D_{obs} = D_f(1 - f_b) + D_{bound}\ f_b \quad (2)$$

where D_f and D_{bound} are the diffusion coefficients of free and micelle-bound enkephalins respectively and f_b is the fraction of the bound peptides. Since the rms displacement of the peptide during the diffusion time allowed in the experiment (typically ∼300 ms) is much larger than the dimension of the micelles, D_{bound} can be taken as equal to the diffusion coefficient of the micelle D_{mic}.

From the calculation of f_b (eq 2) derived from the measured diffusion coefficients, the partition coefficients of the peptides in micelles p, defined as

$$p = [P]_{micelle}/[P]_{aqueous} \quad (3)$$

where [P] is the peptide concentration in the respective phases (assuming the activity coefficients are unity), can be determined. The partition coefficient is related to f_b by the following:

$$[P]_{micelle} = n_p\, f_b/V_{micelle}$$

$$[P]_{aqueous} = n_p(1 - f_b)/V_{aqueous}$$

where n_p is the total number of moles of peptide in the sample, $V_{micelle}$ and $V_{aqueous}$ are the phase volumes of the micelles and the aqueous bulk, respectively, and their ratio is approximated by their respective weight fractions.[13] The detailed procedure and approximation for the determination of the diffusion coefficients and the partition coefficient of peptides in micelles by the PFG-STE diffusion NMR technique was adopted from the previous reports.[13−15,52,53]

For the 1D STD experiments the on-resonance irradiation frequency of the receptor ganglioside GM1 was set to a value of 0.6 ppm where only receptor resonances and no ligand resonances are located. Therefore, in the on-resonance experiment selective saturation of the receptor is achieved. To achieve the desired selectivity and to avoid sideband irradiation, shaped

TABLE 1: Partition Coefficient *P* and Diffusion Coefficients (in 10^{-10} m^2/s) of Free and Bound Enkephalins in 0.3 mM GM1 Micelles at 300 K

	D_f	D_{mic}	D_{obs}	f_b	P
LE	2.731 ± 0.062	0.9056 ± 0.043	1.312 ± 0.035	0.777 ± 0.007	7.68
ME	2.48 ± 0.035		0.986 ± 0.027	0.949 ± 0.001	42.3

pulses are employed for the saturation of the receptor ligand signals. Off-resonance irradiation was set at 30 ppm (1500 Hz), where no receptor signals were present. Internal subtraction of the on-resonance from the off-resonance spectrum leads to a difference spectrum in which only signals of ligand protons are visible that were attenuated via saturation transfer. The irradiation power of the selective pulses in all STD NMR experiments was set to $(\gamma/2\pi) = 90$ Hz. Selective presaturation of the receptor GM1 was achieved by a train of Gauss-shaped pulses of 5 ms length each, separated by a 1 ms delay. A total of 40 selected pulses were applied, leading to a total length of the saturation train of 2.04 or 1.02 s. The spectra were subtracted internally via phase cycling after every scan to minimize artifacts arising from temperature and magnetic field instability. The total scan number in the STD experiments was 128−256, and 16 dummy scans were applied. The spectra were multiplied by an exponential line-broadening function of 2.0 Hz prior to Fourier transformation. All spectra were recorded using a 10 ppm spectral width and a 60 ms spin-lock pulse, which eliminates the background GM1 resonance. Spectral processing was performed on a Silicon Graphics Indy workstation with standard Bruker software.

All NMR samples were prepared in 400 μL of 99.96% D_2O and at 300 K. For STD NMR spectroscopy, the ganglioside GM1 was subjected to two cycles of freeze-drying with D_2O to remove traces of water and transferred in solution to the NMR tube to give a final concentration of 0.3 mM. Addition of enkephalins to the GM1 took place from concentrated stock solutions; therefore, dilution effects were minimal. The stock solutions of LE and ME, 18 mM each, were prepared by dissolving 1.5 mg of LE and 1.6 mg of ME in 99.96% D_2O. The final concentration of enkephalin sample in the NMR tube was 3 mM. The ganglioside GM1 containing a polar and a nonpolar part readily forms a micelle in aqueous solution. The concentration of the GM1 in solution was kept sufficiently high compared to its critical micelle concentration (CMC) of 3.32 μM[54] to ensure the predominance of the micellar aggregates in all experimental concentrations. For PFG diffusion NMR the concentration of free peptide was 3 mM. In the presence of GM1 micelle, enkephalins had the same concentration along with 0.3 mM GM1 giving rise to a lipid/peptide ratio of 1:10.

Results

Diffusion NMR Studies. The variation of the diffusion coefficients of free peptides (D_f), peptides bound to GM1 micelle (D_{obs}), and the diffusion coefficient of the GM1 micelle (D_{mic}) are given in the Table 1. The D_f was determined from the peptide signals in the aromatic region. D_{mic} was determined from the GM1 signal at 1.5 ppm. As the exchange between free and micelle-bound enkephalins is fast in the NMR time scale,[41] an exchange-averaged diffusion coefficient (D_{obs}) is calculated for the solutions. The observed diffusion coefficients for Leu-enkephalin and Met-enkephalin in the presence of GM1 micelle under the same conditions at 300 K are 1.312×10^{-10} m^2/s and 0.986×10^{-10} m^2/s, respectively. From eq 1 and the assumptions described in the Materials and Methods, the fraction of enkephalins bound by the GM1 micelle was estimated and is listed in Table 1. It is clear that the GM1 micelle resulted in

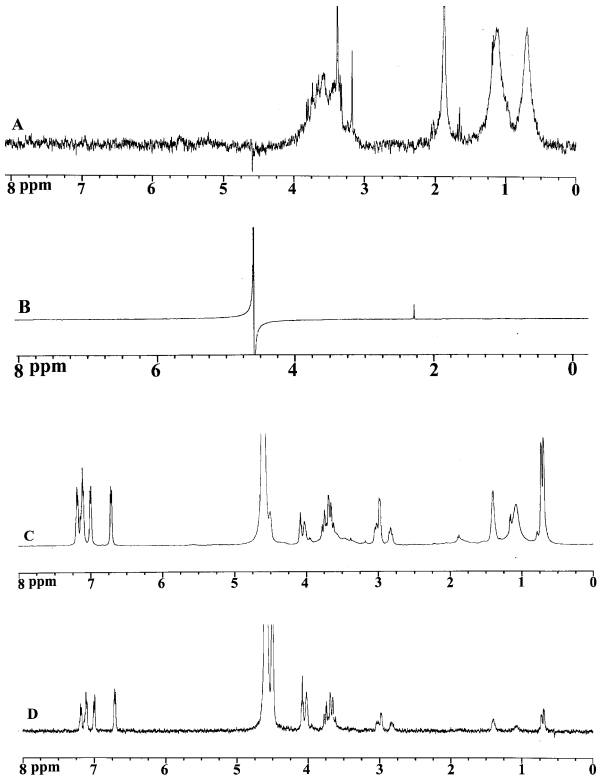

Figure 1. (A) Reference 1D NMR spectrum of GM1 micelle (0.3 mM), displaying the very broad lines normal for a micelle. (B) Corresponding STD NMR spectrum recorded with a $T_{1\rho}$ filter, consisting of a 60 ms spin-lock pulse, to eliminate the resonances of the micelle. (C) Reference 1D NMR spectrum of GM1 micelle (0.3 mM) in the presence of 3 mM Leu-enkephalin ligand. (D) Corresponding STD NMR spectrum showing that Leu-enkephalin yields signals and therefore binds to the receptor.

77.7% and 94.9% binding for the Leu- and Met-enkephalin, respectively. A sharp decrease in the diffusion coefficient of the peptide in the presence of the GM1 micelle was observed. Incorporation of the peptide into the micelles decreases the diffusion coefficient of the peptides. The rate of diffusion of Leu- and Met-enkephalin in the presence of 0.3 mM GM1 micelle is similar. The signals of both the peptides in the presence of the micelle decayed at almost the same rate as that of the micelle signal, indicating that the enkephalins are predominantly bound to or partitioned in the micelles as also has been concluded earlier for the case of substance P in the DPC micelle.[13] From eq 3, it was found that the partition coefficients for the Leu- and Met-enkephalins are 7.68 and 42.3, respectively.

STD NMR Studies. The STD technique works most readily with macromolecules where dipolar coupling of proton spins is extremely efficient because of restricted molecular mobility. We have used the methodology with a more challenging system entailing a molecule whose molecular weight is less than the molecular weight cutoff for the STD NMR but which readily forms micelles that have a large molecular weight. The molecular weight of GM1 is 1.56 kDa and that of its micelle is about 470 kDa,[55,56] which forms a large and heavy system in aqueous solution. The slow micellar motion of this lipid in

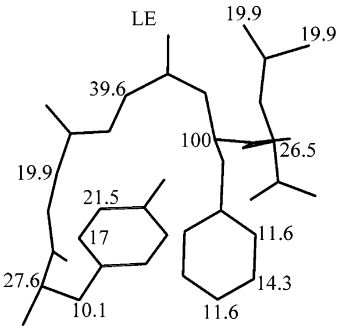

Figure 2. Average solution conformation of Leu-enkephalin in the presence of GM1.[41] Hydrogen atoms have been removed for clarity. The bold numbers on the structure are the STD epitope map obtained by normalizing the largest STD intensity to 100. Table 2 contains the definition of the aromatic protons.

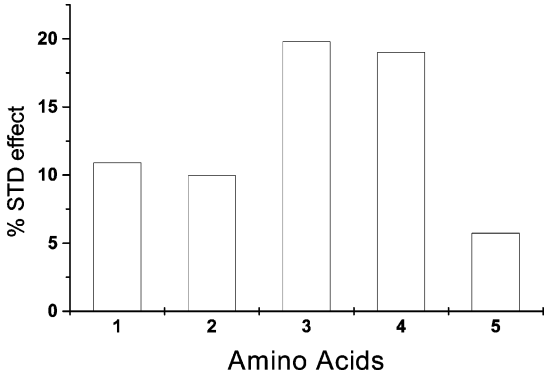

Figure 3. Mean STD values (in percent) of the protons of the individual amino acids calculated for each amino acid of Leu-enkephalin in the presence of GM1 micelle from the 1D STD spectrum.

aqueous solution as well as the high proton density led to a very successful saturation transfer across the entire micelle. Control experiments in the absence of peptide ligand showed complete cancellation of the GM1 signals in the $T_{1\rho}$-filtered difference spectra (Figure 1A,B). When we saturate one signal of a GM1 molecule, through spin diffusion the magnetization is spread over the entire micelle within less than 0.1 s. This saturation of the GM1 micelle is transferred to bound ligands by intermolecular spin diffusion.

TABLE 2: STD NMR Intensities of the Enkephalins in the Presence of GM1 Micelle[a]

resonance	enhancement in LE (%)	enhancement in ME(%)
$Tyr^1\alpha H$	27.67	62.79
βH	10.14	10.1
Ar4H	17.0	11.65
Ar5H	21.59	14.76
$Gly^2\alpha H$	19.94	18.05
$Gly^3\alpha H$	39.61	34.31
$Phe^4\alpha H$	100.0	100.0
βH	7.4	4.2
Ar1H	14.37	8.56
Ar2H	11.65	4.05
Ar3H	11.65	20.68
Leu^5/Met^5		
αH	26.51	×
βH	4.348	2
γH	6.52	6
δH	19.93	
ϵH		11.29

[a] Phe^4Ar1H = ϵH1,2; Phe^4Ar2H = ζH; Phe^4Ar3H = δH1,2; Tyr^1Ar4H = δH1,2; Tyr^1Ar5H = ϵH1,2.

Group Epitope Mapping (GEM). *A. Analysis of Leu-enkephalin.* The responses of the individual protons of Leu-enkephalin have different intensities in the STD spectrum. Figure 1A shows a normal ^{1}H NMR spectrum of GM1 revealing the broad resonances typical for a micelle of this size. Spectrum B in Figure 1 corresponds to the STD NMR spectrum of the same sample with a $T_{1\rho}$ filter consisting of a 60 ms spin-lock pulse prior to acquisition. No GM1 resonance can be detected, since they have relaxed within this period. Figure 1C shows the normal ^{1}H NMR spectrum of ligand Leu-enkephalin, which is not directly affected by the saturation pulse, in the presence of GM1 micelle in a 10:1 ratio. Saturation is exclusively transferred to the molecule bound to the receptor. Therefore, the difference spectrum contains only signals of the molecule with binding affinity. This is shown here in the STD NMR spectra in Figure 1D. Clearly, only NMR signals of the interacting hydrogen of the ligand remain. Use of higher ligand excess is of advantage here because it leads to sharper signals of the exchange-broadened ones. Under these conditions, the ligand STD signals reach higher values and are therefore easily identified. The STD spectrum proves that Leu-enkephalin binds to the GM1 receptor,

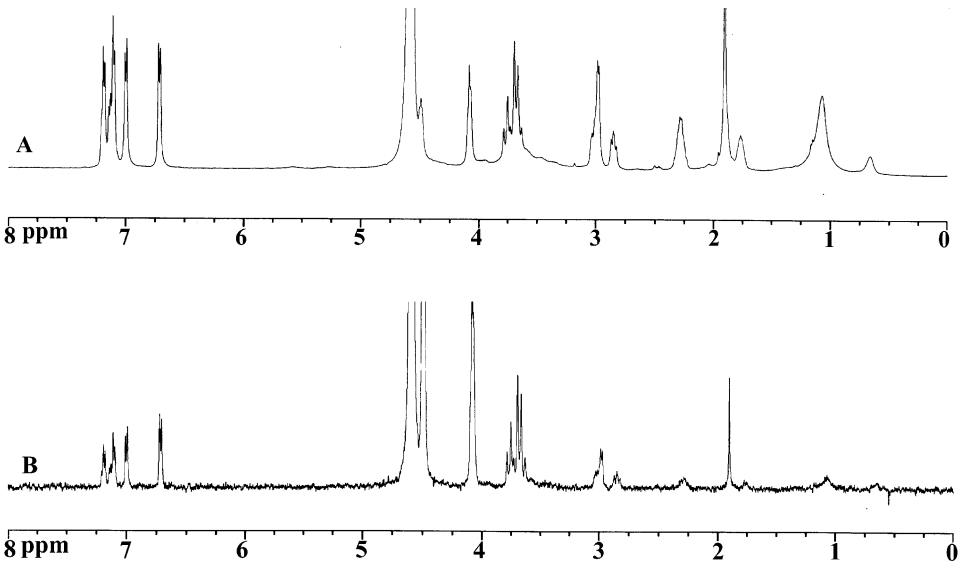

Figure 4. (A) Reference 1D NMR spectrum of 3 mM Met-enkephalin in the presence of ganglioside GM1 (0.3 mM) micelle. (B) STD NMR spectrum of the same sample. Prior to acquisition, a 60 ms $T_{1\rho}$ filter was applied to remove residual micelle resonances.

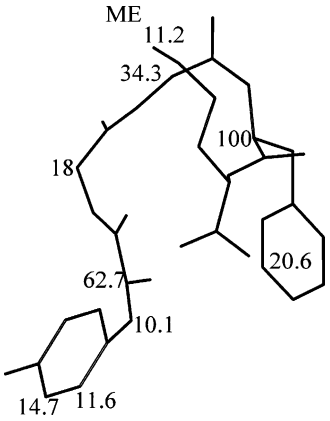

Figure 5. Average solution conformation of Met-enkephalin in the presence of GM1.[41] Hydrogen atoms have been removed for clarity. The bold numbers on the structure are the STD epitope map obtained by normalizing the largest STD intensity to 100. Table 2 contains the definition of the aromatic protons.

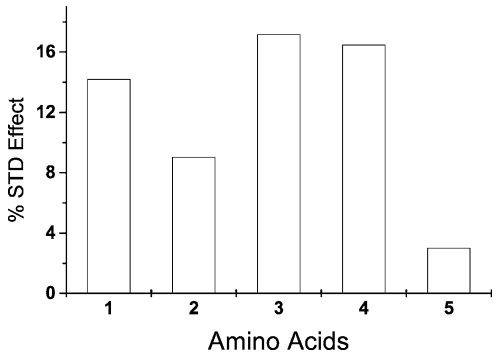

Figure 6. Percent STD effects calculated from the 1D STD of Met-enkephalin in the presence of GM1 micelle. Mean values are shown for the amino acids.

since large signals of the ligand can be observed. In addition, protons of the ligand, which are nearest to protons of the GM1, can easily be identified, because they are saturated to the highest degree. The integral value of the largest proton signal of Leu-enkephalin in the STD spectrum, the Phe^{4}Hα proton, was set to 100%, and those of the other resonances were measured relative to it, to facilitate a comparison of enhancements within the ligand.[23,30] Figure 2 presents the epitope map of 3 mM Leu-enkephalin in the presence of 300 μM of GM1 micelle (with a molar ratio of ligand-to-receptor of 10). Table 2 displays the relative intensities for the individual protons. The Gly^{3}Hα protons have STD intensities in the range of 20% to 60%. On the other hand, all Phe^{4}Ar protons, Tyr^{1}Hα, Tyr^{1}ArH4, 5, Gly^{2}Hα, Leu^{5}Hα, and Leu^{5}Hδ, have similar STD intensities ranging from 10% to 20%. And Phe^{4}Hβ and Leu^{5}Hβ,λ protons have similar STD intensities below 10%. Thus, a clear distinction between protons with strong contact to the receptor, that is, Phe^{4}Hα, and the first series of protons and the others can be made. The mean STD intensities[30] of the each residue are summarized in Figure 3. Here it is evident that proton resonances of the Gly^3 and Phe^4 have the highest intensities in the STD spectrum, signals from the Tyr^1 and Gly^2 are of medium intensity, while the signal of Leu^5 has the lowest intensity. Obviously, Gly^3 and Phe^4 get more saturation from the receptor GM1 than the remaining residues of the ligand and therefore have more and tighter contacts to the lipid micelle.

B. Analysis of Met-enkephalin. Figure 4 shows (A) the normal and (B) the corresponding STD NMR spectra of Met-enkephalin in the presence of ganglioside GM1 micelle. Here also the Phe^{4}Hα proton gives the highest STD integral intensity and was set to 100%. Figure 5 presents the epitope map of 3 mM Met-enkephalin in the presence of 300 μM of GM1 micelle (with a molar ratio of ligand-to-receptor of 10). The relative intensities of the protons are presented in Table 2. The Tyr^{1}Hα, Gly^{3}Hα, protons have STD intensities in the range of 20% to 60%. On the other hand, Tyr^{1}Hβ, ArH4, 5, Gly^{2}Hα, Phe^{4}ArH3, and Met^{5}Hϵ protons have similar STD intensities ranging from 10% to 20%. And Phe^{4}ArH1,2, Hβ and Met^{5}Hβ,χ protons have similar STD intensities below 10%. Relative signal intensity for the bound Met^5 hydrogen is also very poor. The mean of all STD values in each residue is presented in Figure 6 confirming strong interactions of the Gly^3 and Phe^4 group with the GM1 micelle. The signals from Tyr^1 and Gly^2 have medium intensity and, like the leucine moiety, the Met^5 residue also has the lowest STD intensity.

Discussion

We have reported recently the interaction between ganglioside GM1 micelles and neuropeptide enkephalins.[41] However, the 2D NMR data could not provide any information regarding the extent of binding as well as the residues of the peptides that are involved in the binding process. Here we report the altered conformation, diffusion properties, and the partitioning of the peptides in GM1 micelles. Using STD NMR, we have also been able to label the interacting sites of the peptides. Both the experiments were done at a lipid-to-peptide molar ratio of 1:10. Since in both the cases the observed changes are detected from peptides in the free state,[26] a large peptide excess is necessary.[23,25] Increasing the lipid concentration will cause significant line broadening[41] which will make the peak integration difficult. For PFG experiments, the reported lipid-to-peptide ratios are usually higher[13−15,21] than that used in the present study.

The diffusion coefficient of GM1 micelle (at 0.3 mM concentration) was 0.90×10^{-10} m^2/s at 300 K which is within the range of reported diffusion coefficients for micelles (5×10^{-11} to 1×10^{-10} m^2/s).[21] The reported data for the SDS micelle (at 50 mM concentration) is 0.86×10^{-10} m^2/s[57] and that for 228 mM DPC is 0.917×10^{-10} m^2/s.[58] Since the CMC of GM1 is very low (3.32 μM),[54] it is likely that there can be an equilibrium between the GM1 monomeric and micellar forms. In that case the observed D_{mic} may contain contributions from GM1 micelles as well as GM1 monomers. However, measurement of the diffusion coefficient of GM1 monomers will require a solution of GM1 with concentration below the CMC (i.e., less than 3.32 μM),[54] which is too low for NMR measurements. We have estimated that with a 10% smaller value of D_{mic} the f_b's are within 5% of the data reported in Table 1. The use of the D_{mic} value for D_b (the diffusion coefficient of the micelle-bound peptide) has also been reported by Deaton et al.[21] for their pulsed-field gradient NMR study of SDS micelle−peptide association. Similar diffusion coefficients (D_{obs}) have been reported earlier for SDS micelle−Leu-enkephalin[21] (1.24×10^{-10} m^2/s) and for SDS micelle−Met-enkephalin (2.95×10^{-10} m^2/s[59]). The D_{obs} value for Met-enkephalin in zwitterionic micelle DPC is 4.70×10^{-10} m^2/s with an f_b value of 0.59[59] indicating that binding to zwitterionic micelles is less strong.

The diffusion measurement studies presented here have established the strong binding of the peptides with the GM1 micelle. From the high f_b (fraction-bound) value it is evident that both the peptides are strongly bound to the GM1 micelle. Previous results of Palian et al.[60] suggested that Leu-enkephalin analogues reside on the surface of the SDS micelle. For the negatively charged GM1 micelle both the electrostatic and the

hydrophobic interactions might be effective in causing partitioning of the opioid peptides. It should be noted, that the "partition" of the peptides as determined from diffusion measurements does not provide definite information on the exact location of the peptides in the micelles, because "partitioning" or "binding" as defined in the diffusion study requires only that the peptide bind to a micelle and diffuse as a peptide−micelle entity for a period of time longer than the diffusion time (~300 ms). It does not discriminate between partitioning in the interior core of the micelles and binding on the surface of the micelles. It has also been reported that the measured partition coefficient (*P*) using the present technique will have higher accuracy if the fraction $f_b/(1 - f_b)$ is in the range of 0.1 to 10, otherwise a small error in the determination of f_b will lead to an amplified error in the *P* value. In the present study for Met-enkephalin this fraction has a value of ~18.6. Similar high f_b values have been reported for bradykinin (0.99), neurokinin (0.93), and SP (0.96) with SDS micelle.[59]

The STD NMR protocol is based on the transfer of saturation from the receptor to bound ligands.[22,25,26] In macromolecules, dipolar coupling of proton spins is extremely efficient because of restricted molecular mobility. Through spin diffusion the magnetization is spread over the entire receptor within a very small time. This saturation of the receptor is transferred to bound ligands by intermolecular spin diffusion. The irradiation frequency is set to a value where only the receptor resonance and no ligand resonance are located. Therefore, in the on-resonance experiment selective saturation of the receptor is achieved. The signals of the ligands are made visible by subtracting the saturated spectrum from one without receptor saturation. Subtracting a spectrum, where the receptor is saturated from one without receptor saturation, produces a spectrum where only signals of the bound ligands remain in the difference spectrum. Thus, the STD NMR allows identification of binding and characterization of the binding epitopes on the ligands.[22−30]

The group epitope mapping (GEM) by STD NMR described here can be used to characterize at the atomic level binding interactions of ligands with a receptor. Here we demonstrate the method for the binding of two enkephalins to the ganglioside GM1. The results from the STD NMR spectroscopic studies clearly indicate the specificity of the receptor for the hydrophobic portion of the ligand. We observed strong STD signals for amino acids Gly^3 and Phe^4. Tyr^1 and Gly^2 are less strongly bound resulting in smaller integrals. Backbone protons such as $Gly^3H\alpha$, $Phe^4H\alpha$ protons form a tight contact with the receptor. The $H\alpha$ protons of Tyr^1, Gly^2, and Leu^5 as well as the aromatic protons of Phe^4 and Tyr^1 are also involved in binding but with less intense contacts to the receptor. In contrast, the Leu^5 side chain protons show significantly less interactions with the receptor. It is clear from Figure 3 that from the C-terminal part (Leu^5 end) there is little contribution to the binding of the peptide, whereas from the N-terminal part (Tyr^1 end) the binding contribution gradually increases and gives a maximum contribution to the binding at the Gly^3-Phe^4 residue. It was observed that the aromatic residues (Tyr^1 and Phe^4) play a crucial role in binding. The saturation transfer was also efficient for the aromatic protons and indicates that these protons must be close to the receptor surface.

In case of ME, the mean of all STD values in each residue is presented in Figure 6 confirming strong interactions of Gly^3 and Phe^4 with the receptor. They are the most strongly bound site of the ligand. From Figure 6 it is clear that the N-terminal residue Tyr^1 also contributes significantly to the binding. Here the binding integral of the Tyr^1 is much higher than its counterpart in the Leu-enkephalin. All of the aromatic hydrogens give a sufficient amount of STD signal. However, the C-terminal Met^5 residue is very weakly bound to the GM1. Thus, for both the peptides the main interaction is through the Gly^3-Phe^4 region with the C-terminal residue almost out of the binding epitope. It is interesting to note that in our previous report[41] of the three-dimensional structure of LE and ME in the presence of GM1 we observed a turn structure stabilized by a hydrogen bond between the Tyr^1 side chain OH and $Leu^5C{=}O$ and another between Tyr^1OH and Leu^5NH making the Tyr^1 side chain more restricted in LE, whereas, in case of ME, the H bond was between $Tyr^1C{=}O$ and Met^5NH making the Tyr^1 side chain comparatively free. Our STD results also indicate that in the case of ME, the Tyr^1 side chain contributes more than the same amino acid in LE. This is also reflected in the increased partitioning of ME in the GM1 micelle compared to LE as observed from the diffusion studies (Table 1). The observed diffusion coefficient of free LE is 0.25×10^{-10} m^2/s more than that of free ME. The molecular weight of LE and ME being 556 and 574 Da, respectively, it is not unlikely that such differences might be observed. Deaton et al.[21] found that small changes in the molecular weight (even in the case of Gly-Gly and Ala-Gly) might cause such changes in the diffusion coefficient of the free peptide (the *D* value changed by 1.2×10^{-10} m^2/s). However, ligand mobility is not crucial for the ligand binding; the saturation transfer depends on the strength of the binding[22,25,26] rather than any other physical property of the ligand. Hence, the higher partitioning of the ME in the GM1 micelle is more likely due to its increased binding with GM1 through two bulky aromatic side chains, namely, Tyr^1 and Phe^4, in addition to Gly^3. These results also suggest that the primary interaction of the GM1 micelles with these opioid peptides is through the hydrophobic residues and is, therefore, presumably hydrophobic in nature.

Conclusion

The objective of the present study was to investigate the hydrodynamic properties of enkephalins in the presence of a potential intermediate receptor, ganglioside GM1, and to identify the components that take a major role in the peptide−lipid binding. To the best of our knowledge this is the first description of an application of STD NMR spectroscopy to saturate a micelle and determine the epitope of the bound ligand showing the advantages of this direct approach for the purpose of relatively quick and direct epitope determination. The strategic positioning of gangliosides in the nerve cell membranes makes them the molecule of choice to study for any possible interaction between them and other molecules of neurobiological importance. The resent observations of gangliosides binding to a variety of peptides, for example, amyloid β-peptide,[61−64] bee venom melittin,[42] substance P[65], and so forth, motivated the present study. The results of the present study clearly indicate a direct interaction between neuropeptide enkephalins and ganglioside GM1. The interaction is mainly through the central two residues of the peptides indicating a hydrophobic interaction.

Acknowledgment. This work is supported by the Council for Scientific and Industrial Research, Government of India (No. 01(1790)/02/EMR-II). The NMR facilities of the National Facility for 500 MHz NMR at Bose Institute, Kolkata are acknowledged. We are grateful to Dr. Moriz Mayer (University of California), Professor Bernd Meyer (University of Hamburg), and Professor Thomas L. James (University of California) for the STD NMR pulse programs.

References and Notes

(1) Kaiser, E. T.; Kezdi, F. J. *Annu. Rev. Biophys. Chem.* **1987**, *16*, 561−581.
(2) Schwyzer, R. *Chemtracts: Biochem. Mol. Biol.* **1992**, *3*, 347−379.
(3) White, S. H.; Whimley, W. C. *Curr. Opin. Struct. Biol.* **1994**, *4*, 79−86.
(4) Wiese, A.; Gutsmann, T.; Seydel, U. *J. Endotoxin Res.* **2003**, *9* (2), 67−84.
(5) McDonnell, P. A.; Opella, S. J. *J. Magn. Reson.* **1993**, *102*, 120.
(6) Kloosterman, D. A.; Scahill, T. A.; Friedman, A. R. *Pept. Res.* **1993**, *6*, 211−218.
(7) Chen, A.; Wu, D.; Johnson, C. S., Jr. *J. Phys. Chem.* **1995**, *99*, 828−834.
(8) Hicks, R. P.; Beard, D. J.; Young, J. K. *Biopolymers* **1992**, *32*, 85−96.
(9) Deber, C. M.; Benham, B. A. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 61−65.
(10) Graham, W. H.; Carter, E. S., II; Hicks, R. P. *Biopolymers* **1992**, *32*, 1755−1764.
(11) Piconne, D.; D'Ursi, A.; Motta, A.; Tancredi, T.; Temuss, P. A.; *Eur. J. Biochem.* **1990**, *192*, 433.
(12) Zetta, L.; Demarco, A.; Zannoni, G. *Biopolymers* **1986**, *25*, 2315−2323.
(13) Gao, X.; Wong, T. C. *Biopolymers* **1998**, *45*, 395−403.
(14) Gao, X.; Wong, T. C. *Biopolymers* **1999**, *50*, 555−568.
(15) Gao. X.; Wong, T. C. *Biophys. J.* **1998**, *74* (4), 1871−1888.
(16) Chien, W. J.; Cheng, S. F.; Chang, D. K. *Anal. Biochem.* **1998**, *264*, 211−215.
(17) Buchko, G. W.; Rozek, A.; Hoyt, D. W.; Cushley, R. J.; Kenedy, M. A. *Biochim. Biophys. Acta* **1998**, *1392*, 101−108.
(18) Orfi, L.; Lin, M.; Larive, C. K. *Anal. Chem.* **1998**, *70*, 1339−1345.
(19) Yan, J.; Kline, A. D.; Mo, H.; Shapiro, M. J.; Zartler, E. R. *J. Magn. Reson.* **2003**, 270−276.
(20) Whitehead, T. L.; Jones, L. M.; Hicks, R. P. *Biopolymers* **2001**, *58*, 593−605.
(21) Deaton, K. R.; Feyen, E. A.; Nkulabi, H. J.; Morris, K. F. *Magn. Reson. Chem.* **2001**, *39* (5), 276−282.
(22) Mayer, M.; Meyer, B. *Angew. Chem., Int. Ed.* **1999**, *38* (12), 1784−1788.
(23) Johnson, M. A.; Pinto, B. M. *J. Am. Chem. Soc.* **2002**, *124*, 15368−15374.
(24) Moller, H.; Serttas, N.; Paulsen, H.; Burchell, J. M.; Taylor-Papadimitriou, J.; Meyer, B. *Eur. J. Biochem*. **2002**, *269*, 1444−1455.
(25) Mayer, M.; James, T. L. *J. Am. Chem. Soc.* **2002**, *124*, 13376−13377.
(26) Meinecke, R.; Meyer, B. *J. Med. Chem.* **2001**, *44*, 3059−3065.
(27) Kooistra, O.; Herfurth, L.; Luneberg, E.; Frosch, M.; Peters, T.; Zahringer, U. *Eur. J. Biochem.* **2002**, *269*, 573−582.
(28) Rinnbauer, M.; Ernst, B.; Wagner, B.; Magnani, J.; Benie, A. J.; Peters, T. *Glycobiology* **2003**, *13* (6), 435−443.
(29) Germer, A.; Mugge, C.; Peter, M. G.; Rottmann, A.; Kleinpeter, E. *Chem.—Eur. J.* **2003**, *9*, 1964−1973.
(30) Vogtherr, M.; Peters, T. *J. Am. Chem. Soc.* **2000**, *122*, 6093−6099.
(31) Aburi, M.; Smith; P. E. *Biopolymers* **2002**, *64*, 177−188.
(32) Marcotte, I.; Dufourc, E. J.; Ouellet, M.; Auger, M. *Biophys. J.* **2003**, *85*, 328−339.
(33) Wienk, H. L.; Wechselberger, R. W.; Czisch, M.; de Kruijff, B. *Biochemistry* **2000**, *39* (28), 8219−8227.
(34) Bryson, E. A.; Rankin, S. E.; Carey, M.; Watts, A.; Pinheiro, T. J. *Biochemistry* **1999**, *38* (30), 9758−9767.
(35) Shen, M. Y.; Freed, K. F. *Biophys. J.* **2002**, *82*, 1791−1808.
(36) Hakomari, S. I. *Biochem. Soc. Trans.* **1993**, *21*, 583−585.
(37) Yuan, C.; Johnston, L. J. *Biophys. J.* **2000**, *79*, 2768−2781.
(38) Hillbush, B. S.; Levine, J. M. *J. Biol. Chem.* **1992**, *34* (5), 24789−24795.
(39) Levine, J. M.; Beasley, L.; Stallcup, W. B. *J. Neurosci.* **1984**, *4* (3), 820−831.
(40) Myers, M.; Freire, E. *Biochemistry* **1985**, *24*, 4076−4082.
(41) Chatterjee, C.; Mukhopadhyay, C. *Biopolymers* **2003**, *70*, 512−521.
(42) Chatterjee, C.; Mukhopadhyay, C. *Biochem. Biophys. Res. Commun.* **2002**, *292* (2), 579−585.
(43) Iwamori, M.; Nagai, Y. *Biochim. Biophys. Acta* **1978**, *528*, 257−267.
(44) Poddar, S. K.; Thomas, P. D. *Biochim. Biophys. Acta* **1982**, *688*, 453−459.
(45) Dabrowski, J.; Hanfland, P.; Egge, H. *Methods Enzymol.* **1982**, *83*, 69−86.
(46) Acquotti, D.; Sonnino, S. *Methods Enzymol.* **2000**, *312*, 247−252.
(47) Warren, L. *J. Biol. Chem.* **1959**, *231* (8), 1971−75.
(48) Mills, R. *J. Phys. Chem.* **1973**, *77*, 685−688.
(49) Gibbs, S. J.; Johnson, C. S., Jr. *J. Magn. Reson.* **1991**, *93*, 395−402.
(50) Wu, D.; Chen, A.; Johnson, C. S., Jr. *J. Magn. Reson., Ser. A* **1995**, *115*, 260−264.
(51) Johnson, C. S., Jr. *J. Magn. Reson., Ser. A* **1993**, *102*, 214−218.
(52) Dixon, A. M.; Venable, R. M.; Pastor, R. W.; Bull, T. E. *Biopolymers* **2002**, *65*, 284−298.
(53) Romanowski, M.; Zhu, X.; Kim, K.; Hruby, V. J.; O'Brien, D. F. *Biochim. Biophys. Acta* **2002**, *1558*, 45−53.
(54) Basu, A.; Glew, R. H. *J. Biol. Chem.* **1985**, *260* (24), 13067−13073.
(55) Ulrich-Bott, B.; Wiegardt, H. *J. Lipid Res.* **1984**, *25*, 1233−1245.
(56) Cantu, L.; Cotti, M.; Sonnino, S.; Tettamanti, G. *Chem. Phys. Lipids* **1986**, *41*, 315−328.
(57) Orfi, L.; Lin, M.; Larive, C. K. *Anal. Chem.* **1998**, *70*, 1339−1345.
(58) Kallick, D. A.; Tessmer, M. R.; Watts, C. R.; Li, C. Y. *J. Magn. Reson., Ser. B* **1995**, *109*, 60−65.
(59) Whitehead, T. L.; Jones, L. M.; Hicks, R. P. *J. Biomol. Struct. Dyn.* **2004**, *21* (4), 567−576.
(60) Palian, M. M.; Boguslavsky, V. I.; O'Brien, D. F.; Polt. R. *J. Am. Chem. Soc.* **2003**, *125*, 5823−5831.
(61) Choo-Smith, L. P.; Garzon-Rodriguez, W.; Glabe, C.; Surewicz, W. K. *J. Biol. Chem.* **1997**, *272*, 22987−22990.
(62) Matsuzaki, K.; Horokiri, C. *Biochemistry* **1999**, *38*, 4137−4142.
(63) Takahashi, H.; Hirokawa, K.; Ando, S.; Obata, K. *Acta Neuropathol.* **1991**, *81*, 626−631.
(64) Kakio, A.; Nishimoto, S.; Kozutsumi, Y.; Matsuzaki, K. *Biochem. Biophys. Res. Commun.* **2003**, *303*, 514−518.
(65) Goettl, V. M.; Tejwani, A. G.; Neff, N. H.; Hadjiconstantinou, M. *NeuroReport* **1999**, *10*, 513−516.