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Determination of the pK_a of membrane-bound *N,N*-dimethylsphingosine using deuterium NMR spectroscopy

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Abstract

Deuterium nuclear magnetic resonance (^{2}H-NMR) spectroscopy was applied to determine the pK_a of the protein kinase C (PKC) inhibitor, *N,N*-dimethylsphingosine (DMS), when bound to lipid bilayers composed of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC). The quadrupolar splittings from the deuterium labels at the α-and the β-choline positions of the headgroup of POPC responded to the presence of DMS in a manner indicative of an accumulation of cationic charges near the surface plane occupied by the phospholipid phosphate group. Both quadrupolar splittings varied linearly with the amount of added DMS at pH 7.0. Conversely, at pH 10.0 DMS had virtually no influence on either quadrupole splitting, an effect attributed to titration of the dimethyl amino group of DMS to its neutral form. A DMS titration curve was obtained by quantifying the change in the quadrupolar splittings as a function of pH. The pK_a of membrane-bound DMS was extracted from this ^{2}H-NMR data by simulating the quadrupole splitting-titration curve for different values of the pK_a, yielding a pK_a of 8.8 after non-linear least squares fitting.

Keywords: *N,N*-Dimethylsphingosine; Membrane-bound pK_a; pK_a value; NMR, ^{2}H-

1. Introduction

N,N-Dimethyl-D-sphingosine (DMS) is a potent inhibitor of protein kinase C (PKC) [1–4], an enzyme ubiquitous in mammalian systems. Besides its major role in signal transduction, PKC mediates responses to tumor-promoting phorbol esters [5]. Consequently, DMS has been proposed an anti-tumor agent. Merrill and coworkers [6] concluded from analogue studies that the activity towards PKC of DMS, and others of the sphingosine family of compounds, requires, in addition to a chain length of 18 carbons, that there be a positive charge on the amino function.

This dependence of activity on the ionization state is a feature shared by numerous in vivo regulators and pharmacological agents. Many such agents target specific sites on the cytosolic face of the cell membrane or within the cell proper; both cases require that the agent traverse the outer lipid bilayer. The rate of transmembrane 'flip-flop' of a neutral species will be far greater than that of a charged species. Consequently, the pK_a of the agent when it is located within the lipid bilayer becomes a critical point of comparison when rating the efficiencies of homologues or designing new and improved properties.

The pK_a of an amphiphilic ionizable group is most easily determined using surfactant micelles as 'mock' membranes. However, the results obtained may not necessarily reflect physiological conditions. Surfactant micelles and lipid bilayer membranes differ, for example, in their density of chain packing and their radius of curvature. Many physical characteristics will be influenced by such considerations, a recent example being the enthalpy of peptide binding to lipid bilayer surfaces [7]. It seems likely that the same will be true for the equilibrium state of a membrane-bound ionizable group.

We report here a determination of the pK_a of DMS incorporated into lipid bilayer membranes using the ^{2}H-NMR 'molecular voltmeter' approach developed by Seelig

Abbreviations: TLC, thin-layer chromatography; NMR, nuclear magnetic resonance; ^{2}H, deuterium; PKC, protein kinase C; SPN, D-sphingosine; DMS, *N,N*-dimethyl-D-sphingosine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPA, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphate; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; BTP, 3-bis(tris(hydroxymethyl)methylamino)propane; Mes, 2-(*N*-morpholino)ethanesulfonic acid.

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and coworkers [8]. In this technique the quadrupolar splittings in the ^{2}H-NMR spectra of specifically choline-deuterated phosphatidylcholine respond in a characteristic fashion to the presence of any and all membrane surface charges. This behaviour has been used previously to determine the in situ pK_a of membrane surface ionizable groups such as the amino functions of phosphatidylethanolamine and the anaesthetic tetracaine [9]. The ^{2}H-NMR technique enjoys several advantages. First, the amphiphile may be incorporated into lipid bilayer vesicles composed of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), a phospholipid which closely approximates both the acyl chain length and the degree of unsaturation found in natural lipids. Second, the deuterium labels on the choline headgroup of POPC are nonperturbing and, by removing any ambivalence regarding the source of the NMR signal, eliminate the possibility of spectral interference, such as that which complicates pK_a determinations using ^{1}H-NMR [10]. Third, the quadrupolar splittings of specifically choline-deuterated POPC are sensitive only to charges present at the membrane surface, so that observing such a response indicates a specific location for the charged species.

2. Materials and methods

2.1. Materials

D-Sphingosine (SPN) from bovine brain sphingomyelin was purchased from Sigma (St. Louis, MO), as were the following buffers: 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS), 1,3-bis(tris(hydroxymethyl)methylamino)-propane (BTP), and 2-(*N*-morpholino)ethanesulfonic acid (Mes). Citrate and formalin were purchased from BDH (Toronto, ON). Sodium cyanoborohydride was purchased from Aldrich (Milwaukee, WI). POPC-α-d_2 and POPC-β-d_2 were synthesized as described elsewhere [11].

2.2. Synthesis of N,N-dimethyl-D-sphingosine

The synthetic scheme is a modification of that described by Igarashi et al. [2]. 25 mg (83 μmol) of SPN was added to 25 ml methanol/water (1:1, v/v), and warmed to about 52° C to dissolve SPN. 27 μl of formalin (360 μmol formaldehyde) was added, and the solution was stirred under gentle reflux for 2 h or until imination was complete. 20 mg (320 μmol) sodium cyanoborohydride ($NaBH_3CN$) was then added to reduce the imine intermediate, and the reaction mixture was allowed to proceed, with stirring and reflux, for a further 18 h. The products were extracted into an organic phase according to the method of Bligh and Dyer [12] as follows. To the 25 ml methanol/water (1:1, v/v) reaction mixture the indicated quantities of the particular solvents were added in the indicated sequence with thorough mixing of the aqueous and organic phases prior to each subsequent addition: 19 ml methanol, 47 ml chloroform, 31 ml distilled water, and 62 ml chloroform. Final phase separation was facilitated by centrifugation at 5000 rpm for 30 min at room temperature. The aqueous layer was removed and the organic layer was dried by rotary evaporation. The products were then redissolved in 25 ml methanol/water (1:1, v/v) as before and subjected to another round of methylation under identical conditions. After a second Bligh and Dyer extraction the products were chromatographed on a silica acid column (2.5 × 9.4 cm) and eluted with 150 ml chloroform followed by chloroform/methanol (80:20, v/v). The contents of individual fractions were monitored via thin-layer chromatography on silica gel G plates using a carrier system consisting of chloroform/methanol/ammonia (80:20:2, v/v) plus detection using iodine vapours. The R_f values for SPN and DMS were 0.55 and 0.64, respectively. DMS eluted after the first 20 ml of chloroform/methanol (80:20, v/v). The identity of the product was confirmed via ^{1}H-NMR spectroscopy in $CDCl_3$ at 60 MHz (δ 5.2, multiplet, 2H; δ 3.9, pseudo triplet, J = 10 Hz, 1 H; δ 3.3, doublet, J = 10 Hz, 2 H; δ 2.2, multiplet,1H; δ 2.1, singlet, 6H; δ 1.6, multiplet, 4 H; δ 0.9, broad singlet, 22 H; δ 0.5, triplet, 3 H).

2.3. Buffer preparation

All buffer solutions were prepared at a concentration of 50 mM in the buffering species, plus 100 mM sodium chloride. The following buffers were employed in the indicated ranges of pH: citrate (pH 3.0–5.5), Mes (pH 5.5–6.5), BTP (pH 6.5–9.5), and CAPS (pH 10–11). The pH was measured with a microelectrode (Microelectrode, Londonderry, NH, model MI 412) operating from a Corning pH meter calibrated with standard buffer solutions.

2.4. Sample preparation

Appropriate volumes of stock solutions of POPC-α-d_2, POPC-β-d_2, or DMS in chloroform were mixed to achieve the desired final mole percent DMS in 15 mg (19.7 μmol) POPC. Solutions were thoroughly mixed by vortexing, the chloroform was removed under a stream of nitrogen or argon, and any remaining solvent traces were removed under high vacuum overnight. Each dried lipid sample was hydrated with 100 μl of the appropriate buffer by gently warming and vortexing until a uniform suspension was achieved. The samples were then frozen and thawed three times to achieve complete equilibration of the aqueous chambers within the resulting multilamellar lipid vesicles, and the pH was measured once more with the microelectrode. In general, the pH of the buffered solutions decreased by approximately one half a pH unit upon being used to hydrate the lipids. Thereafter, the pH values remained essentially constant as determined from pH measurements following the NMR experiments.

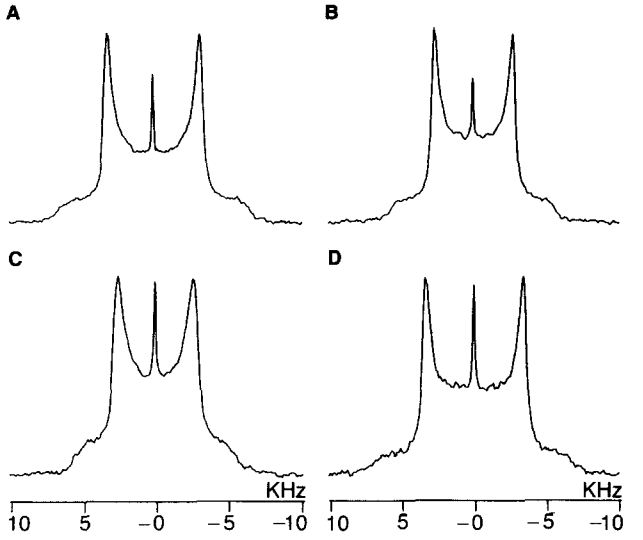

Fig. 1. Deuterium NMR spectra of lipid bilayers composed of POPC-α-d_2 (A and C) or POPC-β-d_2 (B and D), in the absence of DMS (A and B) or the presence of 6.3 mol% DMS (B) and 7.4 mol% DMS (D). The quadrupolar splitting corresponds to the separation in Hertz between the two maxima in the Pake pattern lineshapes. The sharp central spike at 0 kHz arises from the natural abundance deuterium in water. DMS decreases the quadrupolar splitting from POPC-α-d_2 and increases the quadrupolar splitting from POPC-β-d_2. This response indicates that DMS produces a positive membrane surface charge.

2.5. NMR measurements

^{2}H-NMR spectra were recorded on a Chemagnetics CMX300 NMR spectrometer operating at 45.98 MHz, using a Doty broad-line probe equipped with a 10-mm diameter solenoid coil. The quadrupole echo pulse sequence [13] was employed using quadrature detection with complete phase cycling of the pulse pairs and a 90° pulse length of 2.55 μs, an interpulse delay of 40 μs, a recycle delay of 100 ms, a spectral width of 100 kHz, and a 2K data size. Typically, 36000 scans were acquired for each spectrum.

3. Results

3.1. DMS produces a positive charge at the lipid bilayer surface

The effect of DMS on the ^{2}H-NMR spectra from lipid bilayers consisting of POPC-α-d_2 or POPC-β-d_2 are shown in Fig. 1. The overall spectral lineshapes are in all cases typical of lipids incorporated into a liquid-crystalline lipid bilayer. One immediately concludes that, at the molar ratios employed here, DMS does not induce any non-bilayer lipid phases. ^{31}P-NMR spectra of these same samples likewise indicate the absence of any non-bilayer lipid phases (spectra not shown). The quadrupolar splitting corresponds to the separation, in Hertz, between the two maxima in the ^{2}H-NMR Pake pattern lineshape. The size of the quadrupolar splittings for POPC-α-d_2 and POPC-β-d_2 in the absence of DMS (6.3 kHz and 5.5 kHz, respectively) agree closely with values reported previously for neutral lipid bilayer surfaces ([14] and references therein). Addition of DMS at pH 7.0 decreases the quadrupolar splitting obtained from POPC-α-d_2 and increases that obtained from POPC-β-d_2. This is the behaviour expected in the presence of a species which induces a positive surface charge. Furthermore, only one quadrupolar splitting is observed, and there is no evident broadening in the distribution of splittings, demonstrating that the DMS is homogeneously distributed amongst the lipid bilayers and diffuses rapidly, on the ^{2}H-NMR timescale, within the plane of a given lipid monolayer.

According to the molecular voltmeter model of Seelig et al. [8], any charged species located at the lipid bilayer interface has opposite effects on the quadrupolar splitting of the α-versus β-deuterons of the choline headgroup of POPC. Positive surface charges decrease the quadrupolar splitting from α-deuterons and increase the quadrupolar splitting from β-deuterons, while negative surface charges increase the quadrupolar splitting from α-deuterons and decrease the quadrupolar splitting from β-deuterons. This so-called molecular voltmeter effect is only observed for charges located at the polar interface between the lipid bilayer membrane and the aqueous solution. We conclude that DMS binds to lipid bilayer membranes in a fashion such that its dimethyl amino group is protonated and located at the membrane surface.

The quadrupolar splittings from POPC-α-d_2 and POPC-β-d_2 to DMS vary linearly with DMS concentrations over a range towards 20 mol%, as shown in Fig. 2. We did not examine the effects of higher DMS levels for several reasons. First, the sensitivity of the response of the quadrupole splittings to low levels of DMS is quite good and higher levels were deemed superfluous, given that DMS must be synthesized from a rather expensive precursor. Second, previous ^{2}H-NMR studies have shown that non-linearities in the surface charge-quadrupolar splitting response curve are to be expected at higher surface charge densities, a situation detrimental to the determination of pK_a as employed here. Third, given the surfactant-like structure of DMS, higher levels are likely to induce non-bilayer lipid phases and undermine the validity of the entire analysis.

One may describe the relationship between the quadrupolar splitting in the presence of a given amount of DMS, $\Delta\nu_j$, relative to the quadrupole splitting in the absence of added DMS, $\Delta\nu_0$, as a function of the amount of added DMS according to Eq. (1),

$$\Delta\nu_j^i = \Delta\nu_0^i + k^i(DH_j^+) \quad (1)$$

where the superscript i differentiates the α-versus β-deuterons, the subscript j designates a particular overall mol fraction of DMS, (DH^+) is the mol fraction of the cationic form of DMS, and k^i is a proportionality constant deter-

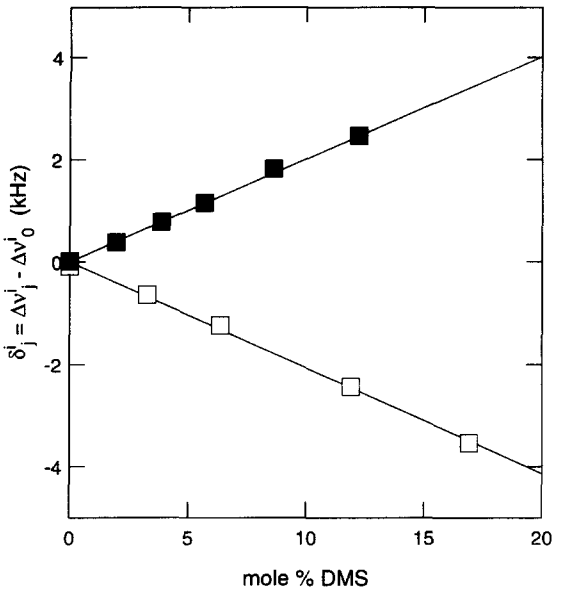

Fig. 2. Variation of the quadrupolar splitting with the amount of DMS. The quadrupolar splittings are expressed as the net difference, δ^i_j, between the quadrupolar splittings measured in the presence, $\Delta\nu^i_j$, versus the absence, $\Delta\nu^i_0$, of DMS at pH 7.0. The superscripts 'i' differentiate the α- versus the β-deuterons, while the subscript 'j' refers to a specific DMS concentration. Open squares represent the α-deuterons and closed squares represent the β-deuterons. The absolute quadrupolar splitting in the absence of DMS was 6.4 kHz for POPC-α-d_2 and 5.5 kHz for POPC-β-d_2. The solid lines show the linear dependence of the quadrupolar splittings on the amount of DMS according to Eq. (1), with slopes obtained from linear regression analysis equal to -21 kHz/mol DMS and $+20$ kHz/mol DMS, respectively, for POPC-α-d_2 and POPC-β-d_2.

mined from the slope of the curves in Fig. 2 using linear regression. For POPC-α-d_2, k^α equals -21 kHz/mol, while for POPC-β-d_2, k^β equals 20 kHz/mol. From the absolute value of the proportionality constant we conclude that the sensitivity of the POPC choline headgroup to cationic DMS is comparable to that observed with other cationic amphiphiles such as didodecylammonium bromide [15]. This comparison would seem to suggest, but does not directly prove, that DMS binds quantitatively to the lipid bilayer membranes at the levels investigated here. Regardless of this question, the sensitivity comparison confirms that DMS is positioned within the lipid bilayer such that the dimethyl amino group is located near the plane occupied by the phosphate group of the phosphocholine headgroup. It seems likely that this depth of penetration of the polar segments of DMS would only be possible if its long alkyl chain were intercalated into the hydrophobic interior of the bilayer proper. It is curious that cationic species in general exhibit a larger influence on the α-segment than on the β-segment, while for DMS the two deutero-segments are about equally sensitive. The magnitude of the ratio of the slopes k^β/k^α is typically 0.5 for cations and 1.0 for anions [15]. Significant deviations from this generalization do occur, however, for cationic amphiphiles such as tetracaine and quaternary ammonium species, where the slopes k^β/k^α approach 0.75. The difference appears to correlate with the amphiphilicity of the cation, suggesting that vertical location within the lipid bilayer interface plays some role in determining the relative sensitivity as well as the absolute sensitivity of the α-versus β-segments slopes. Thus, DMS would appear to penetrate quite deeply into the lipid bilayer.

3.2. Titration of the ^{2}H-NMR response yields the pK_a of membrane-bound DMS

When the pH of the aqueous solution bathing the lipid bilayers is varied in the range from 3 to 12, the ^{2}H-NMR quadrupole splitting from both POPC-α-d_2 and POPC-β-d_2 in the presence of DMS changes progressively as shown in Fig. 3. In the absence of DMS the quadrupolar splittings are virtually independent of the solution pH in this range, with the exception of values below pH 5.0 as discussed further below. The values of the quadrupolar splittings shown in Fig. 3 are expressed as the difference in the quadrupolar splitting with versus without 5 mol% DMS at any one particular pH. At all pH values below 7.0 the quadrupole splittings are characteristic of the presence of cationic surface charges. At all pH values above 10.0 the quadrupole splittings indicate a neutral membrane surface. The pH dependence at intermediate pH values is consistent

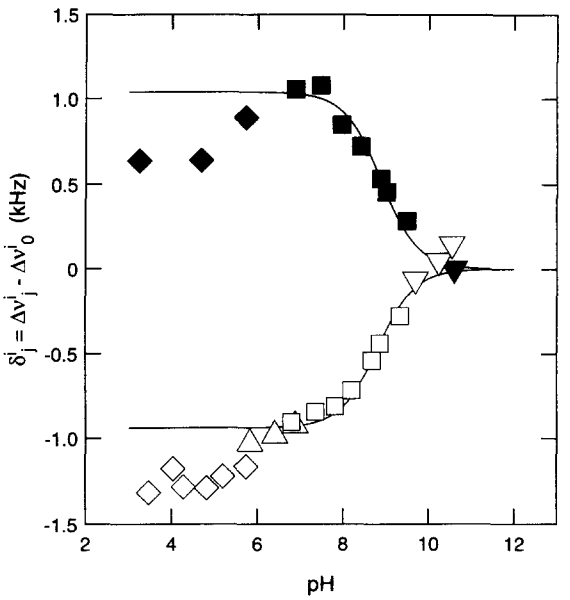

Fig. 3. Experimental and simulated ^{2}H-NMR DMS titration curves. The quadrupolar splittings are expressed as the net difference plus or minus 5 mol% DMS at the indicated pH. The different symbols denote the different buffers used over different ranges of pH: diamond for citrate, triangle for Mes, square for BTP, and inverted triangle for CAPS. Open symbols correspond to experimental values obtained with POPC-α-d_2 and closed symbols to the experimental values obtained with POPC-β-d_2. The solid lines are simulations of the titration curves using Eq. (5) as per the text, with the values of k^α and k^β corresponding to the slopes of the calibration curves in Fig. 2, and the pK_a of DMS equal to 8.8 for both deuteron-labelling positions.

with a titration of a weak base, such as that of the dimethyl amino group of DMS, from its protonated (cationic) form at low pH to a deprotonated (neutral) form at high pH. Consequently, the quadrupolar splitting is a good measure of the degree of protonation of DMS, and provides a means to calculate the corresponding pK_a.

One begins by defining the acid association constant in the usual way as shown,

$$DH^+ \rightleftharpoons D + H^+$$

$$K_a = [D][H^+]/([DH^+]) \quad (2)$$

where D and DH^+ refer to the neutral and cationic (protonated) forms of DMS, respectively. Expressing the total DMS concentration as the sum of these two forms,

$$[T] = [D] + [DH^+] \quad (3)$$

yields the concentration of the cationic form of DMS as a function of pH.

$$[DH^+] = [T]/(1 + (K_a/[H^+])) \quad (4)$$

Substituting Eq. (4) into Eq. (1) permits one to predict the change in the quadrupolar splitting from a given deuteron labelling position and a given pH, assuming a particular pK_a.

$$\delta_j^i = \Delta\nu_j^i - \Delta\nu_0^i = k^i[T]_j/(1 + K_a/[H^+]) \quad (5)$$

where the superscript i again delineates the α-versus the β-deuteron labelling position and the subscript j designates a particular total DMS concentration. The line-of-best-fit, as judged by nonlinear least squares fitting of the predicted to the experimental POPC-α-d_2 and POPC-β-d_2 quadrupole splittings is shown in Fig. 3, and corresponds to the case $pK_a = 8.8$.

In general, Eq. (5) reproduces the pH dependence of the quadrupole splittings in the presence of DMS in a satisfactory manner, with the exception of the low pH end of the titration curve. Below pH 6.0, the quadrupole splittings of the control bilayers lacking DMS abruptly decrease by approx. 0.30 to 0.40 kHz, for both POPC-α-d_2 and POPC-β-d_2. So the effect is not dependent on DMS, but rather on pH. Furthermore, in the presence of DMS, for both deutero-labelling positions, the otherwise smooth change in quadrupolar splittings with decreasing pH is suddenly discontinuous at pH 6.0. So lipid bilayers having bound DMS likewise display the anomaly. Surface charge effects do not explain the behaviour below pH 6.0, since the change in the quadrupole splittings is not in opposite directions for the different deutero-labelling positions. At lower pH values one begins to titrate the phospholipid phosphate, which has been reported to increase the phase transition temperature of phosphatidylcholines [16]. However, an increase in the phase transition temperature should increase motional order parameters and quadrupole splittings, not decrease them as observed here. Moreover, the anomalous behaviour is observed at pH values well above the pK_a of the phospholipid phosphate (approx. 4.0 [16]). It may well be, instead, that we are observing a buffer-specific effect. Notice that in Fig. 3 we have used different symbols to differentiate different buffers. Above pH 6.0 the ionizable group in all buffers used was a weak base, while below pH 6.0 the buffer was citrate, a weak acid. A specific interaction between the lipid bilayer surface and citrate is conceivable and might explain the anomalous ^{2}H-NMR results in this lower pH region. In any case, these effects occur well below the apparent pK_a of DMS and should not unduly influence the analysis presented above.

The equations used to extract the pK_a of membrane-bound DMS are based on several simplifying assumptions deserving some justification. Foremost is the assumption that DMS binding to the lipid bilayers is quantitative at all pH values. The linear dependence of the quadrupolar splittings on the level of added DMS up to nearly 20 mol%, as shown in Fig. 2, strongly suggests that, at the 5 mol% concentration used in the pH titrations, binding levels are far from saturation, at least for the cationic species. The neutral form of DMS should have an even greater affinity for lipid bilayers, but both forms enjoy a hydrophilic/lipophilic balance which strongly favours membrane binding. Thus, one does not expect that the total membrane-bound DMS concentration, [T], should change with pH.

Note further that, at the rather low surface charge densities and high ionic strength employed here, the surface electrostatic potential effect on the DMS binding equilibrium will be minimal. The same applies to the apparent pK_a, which otherwise would be shifted relative to the unperturbed pK_a through the influence of the surface electrostatic potential on the surface pH versus the bulk pH.

In deriving Eq. (5) we have assumed further that the neutral form of DMS has no discernable affect on the ^{2}H-NMR quadrupolar splittings. This is justified by the fact that at high pH the quadrupolar splittings are virtually identical with or without DMS. There are certainly situations where such an assumption would be invalid, in particular where the ionizable amphiphile being titrated constitutes a large fraction of the membrane lipids, or is especially able to alter the lipid bilayer thermotropic or macroscopic phase properties [9]. Neither seems to be the case in the instance of 5 mol% DMS.

4. Discussion

The sphingosines are important regulators in numerous mammalian biochemical pathways, inhibition of PKC being one example. In vivo conversion of SPN to DMS by *N*-methyltransferase has been observed in rat brain [17]. In a series of studies Igarashi and coworkers [1–4] compared

the PKC inhibitory activities of SPN and its *N*-methylated analogues. As opposed to unsubstituted SPN, DMS showed a stronger inhibition of PKC in epidermoid carcinoma A431 cells and B16/BL6 melanoma cells. Most importantly, stereospecificity, a common criterion in the expression of natural compounds, is exhibited by DMS but not SPN. These findings point to a more dominant role of DMS than its parent. Inhibition of PKC has been correlated to a decrease in superoxide generation in circulating neutrophils [18] and interference with the attachment of Lewis lung carcinoma cells to the extracellular matrix [19]. The physiological effects of the sphingosine family are by no means restricted to PKC-dependent phenomena, however. Some of the other affected enzymes include diacylglycerol kinase [20], casein kinase II [21], epidermal growth factor receptor kinase [2], and certain calcium/calmodulin dependent enzymes [22]. Their wide-ranging influences and the pathological conditions associated with their faulty metabolism call for a thorough investigation of the sphingosines at the molecular level.

The pK_a value of 8.8 for DMS calculated by our method is slightly higher than the value of 8.5 for unsubstituted sphingosine measured in surfactant micelles [10]. Differences in experimental conditions aside, the slight upward shift of the pK_a of DMS is readily attributable to an inductive effects of the methyl groups, which would stabilize the positive charge on the protonated species. The same methyl groups prevent DMS from forming the intramolecular hydrogen-bonds between the amino nitrogen and the hydroxyl oxygens on C-1 and C-3 which stabilize the neutral species of SPN.

The differences in the pK_a values of DMS and SPN appear inadequate to explain the differences in their inhibitory effects on PKC. At physiological pH both DMS and SPN will be more than 90% in the protonated form, and either would satisfy the requirement for a positive charge crucial to inhibition of PKC activity. So simply neutralizing the negative charge of the acidic phospholipids required for PKC activity cannot be the sole mechanism through which sphingosines exert their inhibition. The stereospecificity of DMS inhibition of PKC reported by Igarashi et al. [1] supports the notion that a specific interaction is responsible for the inhibitory effects of the sphingosine family, instead of, or in addition to, the general physical mechanism of charge neutralization. Moreover, different subspecies of PKC show different sensitivities to DMS versus SPN. For instance, the potency of DMS observed by Igarashi's group in epidermal carcinoma cell membranes, containing predominantly PKC-α, was not observed by Merrill et al. [6] using PKC derived from rat brain membranes, which contain predominantly PKC-γ [23].

In summary, the pK_a of membrane-bound DMS as determined using ^{2}H-NMR is equal to 8.8, indicating that DMS exists virtually totally in its protonated and charged form at physiological pH. Thus, the capacity of DMS to neutralize anionic membrane surface charges is approximately equal to that of SPN.

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