Physical Properties of the Transmembrane Signal Molecule, sn-1-Stearoyl 2-Arachidonoylglycerol

ACYL CHAIN SEGREGATION AND ITS BIOCHEMICAL IMPLICATIONS*

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Jan-Ove Hindenes‡‡, Willy Nerdal‡‡, Wen Guo**, Li Di‡‡‡, Donald M. Small**, and Holm Holmsen‡‡

From the **Department of Biophysics, Boston University School of Medicine, Boston, Massachusetts 02118, the ‡Department of Biochemistry and Molecular Biology, University of Bergen, Bergen, and ‡‡Department of Chemistry, University of Bergen, N-5007 Bergen, Norway

The abbreviations used are: DAG, sn-1,2-diacylglycerol; PKC, protein kinase C; PIP, phosphatidylinositol 4-phosphate; PIP2, phosphatidylinositol 4,5-bisphosphate; DSC, differential scanning calorimetry; DO, sn-1,2-dioleoylglycerol; SAG, sn-1-stearoyl-2-arachidonoylglycerol; MAS, magic angle spinning; PS, phosphatidylinerine; PLC, phospholipase C; SLG, sn-1-stearoyl-2-linoleoylglycerol.

Although minor components of biological membranes, the sn-1,2-diacylglycerols (DAGs) play pivotal roles in lipid metabolism and cell signaling. DAGs produced from glycerol 3-phosphate in the de novo glycerolipid synthesis are further converted to phosphatidylycholine and phosphatidylethanolamine by the choline and ethanolamine phosphotransferase reactions or converted to triacylglycerol in acyltransferase actions (1). DAGs are also formed during agonist-induced cell activation from preformed glycerophospholipids through (phosphoinositide-specific) phospholipase C and indirectly through the combined action of phospholipase D and phosphatidate phosphohydrolase (2), and from ceramides by sphingomyelin synthase (3). The DAGs formed from preformed phospholipids act as activators of protein kinase C (4), as modulators of phospholipase A2 (5, 6), as regulators of CTP-phosphocholine cytidylyltransferase (1), and are thought to interfere with arachidonate-induced modulation of the GTPase-activating protein activated GTPase of p21(7). The phospholipid-derived (and perhaps also the de novo synthesized) DAGs are also converted by DAG kinase to phosphatidic acid which may be further converted to phosphatidylinositol, phosphatidylglycerol, and cardiolipin (1), or the DAGs are degraded by DAG and monoaoylglycerol lipases to yield free fatty acids, of which arachidonate is utilized for eicosanoid synthesis (8–10). DAGs also appears to facilitate translocation of enzymes to membranes, as discussed for protein kinase C (PKC) (11), dicylglycerol kinase (12–14), and phosphocholine cytidylyltransferase (CTP) (15).

In many of these conversions and actions, the exact molecular species of the DAGs appears to be important, indicating a high degree of acyl specificity of the enzymes involved, particularly in the polyphosphoinositide cycle where some isoforms of DAG kinase (16–19) and CDP-sn-1,2-diacylglycerol synthase (19) are highly specific for sn-1-stearoyl-2-arachidonoylglycerol (SAG), which explains the great abundance of sn-1-stearoyl-2-arachidonoyl species among (mammalian) phosphatidylinositol, phosphatidylinositol 4-phosphate (PIP), and phosphatidylinositol 4,5-bisphosphate (PIP2) (20, 21). Moreover, upon thrombin stimulation of platelets many molecular species of DAG are formed, but the concomitant PKC-induced phosphorylation of pleckstrin only correlates with the transient, large accumulation of SAG, showing that at least in intact platelets the SAG species specifically activates PKC (22). Furthermore,
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DAG lipases from many sources use SAG as the preferred DAG species (8–10). Finally, incubation of fibroblasts with radioactive sn-2-arachidonoylglycerol yields mostly radioactive SAG and sn-1-1-stearoyl-2-arachidonoylglycerol-containing lipids (23). sn-1,2-DAGs, but not sn-1,3-DAGs, stimulate Ca\(^{2+}\)\,-induced fusion of phosphatidylserine-containing vesicles (24); sn-1,2-dioleoylglycerol promotes Ca\(^{2+}\)\,-induced fusion of chlamyphagen granules with other membranes (25), and several DAG molecular species facilitate exocytosis of amylase from parotid gland secretory granules (26) and cause an L\(_m\) to H\(_{II}\) transition in several glycerophospholipids at very low mol % of DAG (27, 28). Treatment of phospholipid vesicles with phospholipase C, and thereby forming DAG within the membranes, also causes vesicle fusion (29–31) that is accompanied by formation of DAG-rich domains (30); such domains have also been reported to form during temperature-induced phase transitions of mixtures of DAG and certain phospholipids (32). sn-1,2-Dioleoylglycerol has also been shown to promote binding of protein to phospholipid bilayers (33). Unfortunately, none of these studies employed SAG but were mostly performed with DAGs containing the same fatty acid in both the sn-1 and the sn-2 positions, an acyl combination that is rare in biological glycerophospholipids.

Evidently, SAG appears to be the molecular species of DAGs that displays several pivotal roles in cellular signal transduction and metabolism, and sn-1,2-DAGs (in general) are fusions that may participate in many cellular processes that may involve their tendency to form separate domains and promote L\(_m\) to H\(_{II}\) transition in membranes. It is, therefore, likely that these actions of the DAGs are due to their physical behavior. Previous differential scanning calorimetry (DSC) and x-ray powder diffraction studies have shown that DAGs with the same saturated fatty acid (C\(_{12}\), C\(_{16}\), and C\(_{18}\)) in the sn-1 and -2 positions have a stable bilayered \(\beta\) phase (34–36) that transitions to a metastable \(\alpha\) phase with hexagonally packed chains on cooling below the melting point of the \(\beta\) phase (36). In contrast, the biologically occurring sn-1-stearoyl-2-oyleoylglycerol packs with very complex chain conformations, disorder and marked polymorphism giving rise to at least eight phases in the solid state (37). The sn-1,3 isomer of this DAG, which does not participate in cellular activities, packs in four phases. In the most stable \(\beta\) form the acyl chains come off the glycerol backbone in a 94° V-shape (38), quite unlike the hairpin conformation typical for the acyl groups in the bilayer membrane. The naturally occurring sn-1-stearoyl-2-linoeoylglycerol packs somewhat more efficiently than the sn-2-monounsaturated DAG in \(\alpha\), sub-\(\alpha\), sub-\(\alpha\), and \(\beta\) phases with the \(\beta\) and \(\alpha\) phases thought to have a tilted bilayer and extended bilayer structures, respectively (39).

In the present study we have performed similar calorimetric and x-ray experiments of SAG and complimented these with solid state high field proton decoupling and cross-polarization, magic angle spinning \(^{13}\)C nuclear magnetic resonance studies. We find that SAG exists in \(\alpha\), sub-\(\alpha\), sub-\(\alpha\), phases but no \(\beta\) phase. However, to our surprise, our NMR results show that the stearoyl chain freezes before the arachidonoyl chain on cooling and that the terminal methyl group of the arachidonoyl chains exists in a different milieu than that of the stearoyl chains. These observations together with our finding that the average (001) strong x-ray reflections is around 62–63 Å in the \(\alpha\) phases, in contrast to 55 Å in sn-1,2-distearoylglycerol (36), perhaps suggest that SAG exists in a V-shaped segregated bilayer.

**EXPERIMENTAL PROCEDURES**

sn-1,2-SAG (claimed to be 99% pure) were obtained from Serdary Research Laboratories (Englewood Cliffs, NJ) and were purified by flash column chromatography (under argon) on silica gel (grade 60, 230–400 mesh, Merck) containing 9% boric acid (w/w) to remove about 5% 1,3-sn-SAG and some high RI impurities (UV-sensitive). Eluting solvent (degassed under vacuum and saturated with argon) was either 8% ethyl acetate in hexane or 1–5% acetone in dichloromethane. Fractions were tested for boric acid content by TLC on carboxylic acid-activated 25 Å silica gel hard layer organic binder on a glass support, soaked in 5% boric acid at room temperature, air-dried overnight, and activated for 1 h at 100 °C, eluted with 5% acetone in dichloromethane. Pure fractions were combined and filtered to remove silica dust. All samples were demonstrated to be homogeneous by analytical two-dimensional TLC (boric acid-tert-butanol in hexane for the DAB and 20°C) or dry (rotary-evaporated under reduced pressure, and lyophilized for 15 h in an ice-water bath).

**Differential Scanning Calorimetry (DSC) Experiments**—DSC of dry sn-1,2-SAG was carried out on a Perkin-Elmer DSC-7. Each sample (1.5–5.0 mg), weighed to the nearest 0.01 mg, was sealed (under argon) in a stainless steel pan, and a pan with argon was used as a reference sample. Heating and cooling rates were 5 °C/min. The enthalpies of melting (\(\Delta H_m\)) and crystallization (\(\Delta H_f\)) were determined by using 7 series Microcal software. The transition temperatures were determined from high purity standard material (Indium). Estimated error margin for this standard is less than 0.5 °C in the transition temperature and 2% in enthalpy. Hydrated sn-1,2-SAG samples were made by adding different amounts of water, heating to 35 °C, and shaking for 15 min to equilibrate water with lipid. The amount of water was estimated gravimetrically. All samples were checked for hydrolysis and 1,3-acetyl migration by TLC (41) and no hydrolysis or migration was detected.

**X-ray Powder Diffraction**—X-ray powder diffraction patterns were recorded using nickel-filtered CuK\(_\alpha\) radiation from an Elliot GX-6 (Elliot Automation, Borehamwood, UK) rotating-mode generator equipped with cameras using Franks double-mirror optics (42) and toroidal-mirror optics. The samples were packed into 0.7 or 1.0-mm diameter LCM capillaries (Charles Supper, Natick, MA) and examined in variable temperature sample holders. The rate of cooling and heating to a fixed temperature is 2–4 °C/min. The photographs were taken on a Toroid camera. The diffraction patterns were recorded using thermal programming similar to those used in the DSC investigation.

**NMR**—High resolution \(^{1}H\) and \(^{13}\)C NMR were recorded on a Bruker 500-MHz spectrometer, and solid state \(^{13}\)C NMR values were recorded on a Bruker AMX-300 NMR spectrometer (75 MHz for \(^{13}\)C), equipped with a BLT MAS probe and a high power amplifier unit. Samples were packed under argon in 7-mm Zr\(_2O\) rotors with an insert (sample volume \(\sim 150 \mu l\)). Sample spinning rates were 4.0 kHz for solid state and 20 kHz in isotropic state, and a duty cycle of \(\leq 1.5%\) was used for all experiments. For solid state samples, single contact cross-polarization from the \(^{1}H\) reservoir to \(^{13}\)C spins was employed to increase the \(^{13}\)C sensitivity and to shorten the effective \(^{13}\)C relaxation time (43). Different contact times were used to differentiate the molecular motions. The CH group of adamantane (38.3 ppm) was used as an external chemical shift reference. For hydrated sn-1,2-SAG, water was added, and the sample was heated to 35 °C and mixed vigorously (30 min) before transferring the sample to the rotor. All samples were checked by TLC and \(^{1}H\) NMR (both in CD\(_3\)OD and CDCl\(_3\)). No sn-1,3-SAG, hydrated or oxidized impurities, or trace amounts of solvents were detected. After the solid state NMR experiments, the samples were checked again, and no changes had occurred for the dry sample. Slight isomerization to sn-1,3-SAG (1%) was found for the hydrated sample after 3 days.

**RESULTS**

**Differential Scanning Calorimetry**—Solvent crystallization of sn-1,2-SAG at \(-20 °C\) in hexane, pentane, acetone, and acetonitrile all gave \(\alpha\) phases, and no \(\beta\) phase or \(\beta\) phase values were obtained. The thermodynamic data for dry and hydrated sn-1,2-SAG are shown in Table 1. There are three phases found for dry sn-1,2-SAG as follows: \(\alpha\), sub-\(\alpha\), and
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| Phase/sample | Tm \(^{\circ}C\) | ΔH \(^{kcal/mol}\) | S' cal/mol | Tc \(^{\circ}C\) |
|--------------|-------------|----------------|-------------|-------------|
| Dry sn-SAG   | α           | 7.2            | 8.0         | 28.6        | 3.3         |
|              | Sub-α\(_1\)* | −0.1           | 0.5         | 1.9         | −3.1        |
|              | Sub-α\(_2\) | −5.3           | 1.4         | 5.4         | −8.8        |
| Hydrated sn-SAG | α           | 6.8            | 7.7         | 27.4        | 3.1         |
|              | Sub-β\(_1\) | −1.7           | 0.2         | 0.8         | −4.5        |
|              | Sub-β\(_2\) | −6.3           | 1.1         | 4.2         | −9.5        |
| Arachidonic acid | β           | −37.7          | 7.5         | 24.1        | −68.4       |

* Temperature at melting point, transition peak value.

\(^{b}\) Enthalpy of melting.

\(^{c}\) Temperature of crystallization, transition peak temperature.

\(^{d}\) Polymeric transition process, not melting.

The polymorphic transition process of arachidonic acid is described as a somewhat disordered, two-dimensional rectangular packing of the chains.

The nature of the two sub-α phase transformations is probably a disorder to order process involving conversion of some gauche or skewed bonds to trans-conformation as the temperature is lowered. Since the enthalpies of α to sub-α, and sub-α\(_1\) to sub-α\(_2\) transitions are small, one can estimate that around 15–20% of the gauche bonds in the α form have been transformed to trans bonds in the sub-α\(_1\) form.

The average (001) reflections of the α and sub-α phases are 62.1 to 63.6 Å. The α phase of sn-1,2-distearoylglycerol is about 55 Å. In this phase the chains are presumed to be nearly perpendicular to the bilayer plane. This structure is based on the (001) diffractions of a homologous series of saturated chain sn-1,2-di(iso)acylglycerols (36). This gives an increment of about 1.27 Å per carbon. We have noted that the α phase of sn-1-stearoyl-2-linoleoylglycerol (SLG) is 59.5 Å, thus significantly greater than the distearoyl analog. We argued (38) that the most likely packing of the chains in the α phase was a bilayer with extended acyl chains and an extended glycerol lying perpendicular to the plane of the bilayer. The argument that SLG α phase is a bilayer was bolstered by the fact that the phases hydrate with half a molecule of water that increases the spacing by almost 2 Å. The spacings of the sn-1,2-SAG are about 2 Å longer than the linoleoyl analog and could be consistent with a similar structure, that is a bilayer with extended chains and the glycerol and its sn-1 ester group lying perpendicular to the plane of the bilayer. However, sn-1,2-SAG with water shows no significant change in long spacing and thus appears not to swell with minor hydration. We emphasize, however, that without a true crystalline structure it is difficult to be certain about the meaning of the (001) spacings. In fact, as we noted with the SLG, the intensity distribution in the first, second, and third order do not follow an ordinary bilayer distribution, since the (002) reflection is quite strong. This is also true in the sn-1,2-SAG spacings. Data in this paper may suggest a chain segregated model.

Amount of Bound Water in sn-1,2-SAG—Liquid sn-1,2-SAG was put in a capillary cylinder (8 μcm, Wilmad) mixed with water (30% by volume), and sealed. The contents were mixed vigorously by centrifugation. The capillary cylinder with sample was then inserted in a 5-mm NMR tube containing CDC\(_13\)/tetramethylsilane. Comparison of 1H NMR spectra between the neat liquid of sn-1,2-SAG and sn-1,2-SAG/water at 20 °C shows that there is an extra peak at 4.64 ppm (equivalent to 1 proton), which is different from the unbound water (spectra not shown). Therefore, on a time average, every sn-1,2-SAG molecule binds to half a water molecule in the liquid state. The free –OH group of sn-1,2-SAG shifts from 4.21 to 4.04 ppm upon forming a water complex. 13C NMR shows that the chemical shifts changes for the sn-3-glycerol carbon, –CH\(_2\)-OH, and the sn-2-glycerol carbon, CH\(_2\)-OCO–, shifts upfield by 0.09 ppm after forming hydrated product. The 13C resonances of two carbonyl groups (C=O) shift downfield by 0.14 ppm (sn-1) and 0.17 ppm (sn-2). These changes indicate that the polar head group is involved in the formation of the hydrated compound.

Solid State 1H NMR of 1,2-sn-SAG—Due to very large direct dipole-dipole interaction between abundant nuclei in solids, the 1H spectra of the different phases of sn-1,2-SAG are very broad and do not contain high resolution information, although each different phase has its own characteristic shape (different relative intensity). They all contain five major peaks, tentatively assigned from the isotropic spectra and the solution spectra of sn-1,2-SAG/CDC\(_13\)/H\(_2\)O–C= C (5.8–4.7 ppm), =C=CH\(_2\)-C= (3.3–2.2 ppm), –CH\(_2\)-C= (2.4–1.8), –CH\(_2\)= (1.5–0.9), and the methyl peak (–CH\(_3\)) (0.9–0.5 ppm). Upon hydration (spectra not shown), two additional peaks appear in the α phase, one for unbound water (4.9 ppm) and one broad peak for water bound to sn-1,2-SAG (3.6 ppm). In the sub-α\(_1\) phase both the 4.9 ppm peak (unbound water) and the broad 3.6 ppm peak (bound
FIG. 1. Differential scanning calorimetry of SAG at cooling (a) and heating (b) rates of 5 °C/min. The curve in a shows that the SAG melt enters the solid α phase at \( T_c = 3.3 \) °C. Upon further cooling, \( \alpha \) will transform to sub-\( \alpha_2 \) (\(-3.1 \) °C), and sub-\( \alpha_2 \) will transform to sub-\( \alpha_2 \) (\(-8.8 \) °C). The curve in b shows that SAG in sub-\( \alpha_2 \) will transform to sub-\( \alpha_1 \) at \(-5.3 \) °C, and sub-\( \alpha_1 \) will transform to the \( \alpha \) phase at \(-0.1 \) °C. By further heating, the \( \alpha \) phase will melt at 7.2 °C. Thus, \( \alpha \) can transform to sub-\( \alpha_1 \), and then sub-\( \alpha_2 \), reversibly. Table I lists the thermodynamic data from curve b, which also defines the \( \alpha \), the sub-\( \alpha_1 \), and the sub-\( \alpha_2 \) phases used throughout the paper.

### Table II

| Phase | \( \alpha \) | Sub-\( \alpha_1 \) | Sub-\( \alpha_2 \) |
|-------|------------------|------------------|------------------|
| α     | 62.1 (av)        | 62.8 (av)        | 63.6 (av)        |
| 62.2 (s), 1 | 62.8 (vs) | 65.5 (vs), 1 | 60.8 (m), 1 |
| 30.9 (s), 2  | 31.4 (vs), 2 | 31.8 (vs), 2 | 30.4 (m), 2 |
| 20.8 (w), 3  | 20.8 (w), 3 | 15.6 (m), 4 | 15.9 (w), 4 |
| 4.28 (s)  | 4.33 (s) | 4.31 (vs) | 4.29 (m) |
|        | 4.00 (m) | 3.83 (s) | 3.96 (m) |

- **Av.**, average 001 spacing calculated from all the orders. Relative intensity is given in parentheses as follows: vs, very strong; s, strong; m, medium; w, weak. The order of 001 reflection is indicated after the diffraction long spacing intensity.

- **Solid State \(^{13}\text{C} NMR of sn-1,2-SAG**—Scheme 1 presents the SAG atom numbering. The various types of carbons in SAG are found in distinct chemical shift regions (Fig. 2). They are as follows: the two carbonyl carbons (s1 and a1), the eight double bonds carbons in the arachidonoyl moiety (a5–a6, a8–a9, a11–a12, and a14–a15), the carbons adjacent to double-bonded carbons (a4, a7, a10, a13, and a16), the three carbons of the glycerol backbone (g1, g2, and g3), the carbons with single bonds to carbons in both the stearoyl (s2–s17), and the arachidonoyl (a2–a3 and a17–a19) chains and the chain terminal methyl group of the stearoyl (s18) and the arachidonoyl (a20) moieties. However, assigning each of the SAG carbon resonances is in some cases difficult just based on chemical shifts. Therefore, differences in the carbon mobilities of the stearoyl and arachidonoyl moieties in the different phases are used to assist the assignments of the carbon resonances. Figs. 3–6 present high power proton-decoupled \(^{13}\text{C} \) spectra of SAG in the isotropic phase and the solid phases \( \alpha \), sub-\( \alpha_1 \), and sub-\( \alpha_2 \). These figures show the carbonyl spectral region (Fig. 3), the glycerol backbone spectral region (Fig. 4), the C=C spectral region (Fig. 5), and the CH2 and CH3 spectral regions (Fig. 6).

- **Assignments of Carbonyl and Glycerol Carbon Resonances**—Assignments of the carbonyl resonances (Fig. 3) are based on earlier assignments of these carbons in dioacylglycerols (46), and the glycerol backbone carbon resonance g2 (Fig. 4) is assigned according to published chemical shift value (47). The differentiation of the glycerol g1 and g3 carbon resonances (Fig. 4) is tentative and based on expected differences in shielding, assuming that the hydroxyl group attached to g3 provides stronger shielding than the ester group attached to g1. However, these glycerol backbone assignments (Fig. 4) are further supported by differences in mobility of the g1 and g3 carbons. The g1 carbon carrying the stearoyl moiety experiences the higher reduction in mobility of the three glycerol backbone carbons when SAG enters the solid phase \( \alpha \) from the isotropic phase as compared with g2 and g3. This is confirmed by the observed reduction in mobility of the stearoyl carbons upon the isotropic to solid phase \( \alpha \) transition (see below).

- **Carbon with Double Bonds**—The eight C=C carbon resonances of the arachidonoyl moiety in the four phases studied are presented in Fig. 5. The assignments of these eight resonances are close and are correlated (45). In general, the carbon resonances of SAG displayed in Figs. 3–6 experience an upfield shift when the sample is brought from the isotropic phase to the solid phase. Chemical shift values under the assumption that the chemical shift values between isotropic phase and the three solid states are closely correlated (45).
nances are still tentative and appear on top of the carbon peaks in the isotropic phase spectrum, see Fig. 5A. Studies (48) on mixtures of triacylglycerols and fatty acids containing oleate, linoleate, and linolenate (in CDCl₃ solution) show that C=C resonances usually appear as two peaks with the carbon furthest away from the glycerol moiety (highest carbon number in the acyl chain) as the most downfield resonance. The resonances experience an upfield shift from the isotropic to the solid phase as spectrum (Fig. 5B). Assuming that the higher degree of mobility in the arachidonoyl chain is in the tail (methyl end), carbon resonances a₁₅ and a₁₄ can be identified in the spectrum of SAG in sub-α₁ phase, see Fig. 5D. Further assuming that the least mobile of the eight C=C arachidonoyl carbons are a₅ and a₆, these resonances can be identified as the C=C pair most affected by the loss of mobility in the arachidonoyl moiety as SAG is brought from the isotropic phase through the solid phases a, sub-α₁, and sub-α₂ phases. These resonances are labeled with the arachidonoyl carbon number, and the peak assigned to the three carbons of the arachidonoyl chain residing between two carbons with double bonds is labeled a₇, a₁₀, and a₁₃. In the sub-α₁ and the α phases, Figs. 5C and 5B, respectively, this peak has about three times the intensity of the other single resonance peaks, thus supporting the assignment of a combined peak for the a₇, a₁₀, and a₁₃ carbons. Furthermore, in the sub-α₂ phase this peak has about 1.6 times the intensity of the a₁₆ peak, indicating that the a₇ resonance does not contribute to the peak intensity. i.e. the arachidonoyl chain has lost mobility in the carbon 1–7 chain at −27 °C. Further support of the assignment of the combined a₇, a₁₀, and a₁₃ peaks comes from studies on mixtures of triacylglycerols and fatty acids containing oleate, linoleate, and linolenate (in CDCl₃ solution) by ¹³C NMR (48). It is found that the chemical shift value of a CH₂ carbon in a position to two cis double bonds is 25–26 ppm,
which corresponds well to the 25.9 ppm peak assigned to carbons $a_7$, $a_{10}$, and $a_{13}$ in Fig. 6A. The chemical shift ranges of other acyl chain carbons are found to be about 33.8 ppm for acyl-C2, about 25 ppm for acyl-C3, about 27 ppm for a CH$_2$ carbon in $a$ position of one cis double bond, about 32 ppm for acyl-C($n - 2$) and about 22.7 ppm for acyl-C($n - 1$). All these chemical shift values are in good agreement with the corresponding assignments of these carbon resonances in Fig. 6A. The differentiation of two corresponding carbon resonances in the stearoyl and arachidonoyl chains is based on the observed differences in mobility of these two moieties. This has also been done in the assignments of the two CH$_2$ carbons in $a$ position to one cis double bond, i.e. the resonances $a_{16}$ and $a_4$, in that only the former shows mobility in the sub-$a_2$ phase.

Resonances of CH$_2$ Carbons in Direct Chain—In spectra B and C of Fig. 6 a broad CH$_2$ peak is present at 32.5 ppm, confirming the crystalline state of these CH$_2$ carbons (47). The isotropic phase (Fig. 6A) chemical shift of this peak is 30.4 ppm, and this peak originating from CH$_2$ groups in the stearoyl moiety is labeled s(1/2)$CH_3$. The described behavior of these stearoyl CH$_2$ resonances in the $a$ phase (crystalline state of the stearoyl moiety), where the arachidonoyl CH$_2$ resonances appear as “liquid crystalline” state resonances (carbons that have higher mobility than those in the crystalline state), demonstrates the different mobilities of the stearoyl and the arachidonoyl moieties. Furthermore, this differentiated mobility of the stearoyl and the arachidonoyl moieties is also present in the sub-$a_1$ and sub-$a_2$ phases (Fig. 6, C and D). In the sub-$a_2$ phase the stearoyl CH$_2$ resonances vanish due to impaired mobility, see Fig. 6D. Thus, as the temperature is lowered, the stearoyl chain freezes before the arachidonoyl chain.

Acyl Chain Terminal CH$_3$ Groups—Additional support of the differential mobilities of the stearoyl and the arachidonoyl moieties is found in the behavior of the terminal methyl resonances of the two acyl chains. This resonance is labeled $s_{18}$ and $a_{20}$ in the isotropic phase spectrum (Fig. 6A), and in the solid phase $a$ and sub-$a_1$ (Fig. 6, B and C) spectra the methyl resonance of the arachidonoyl chain ($a_{20}$) is found upfield of that of the stearoyl chain ($s_{18}$). The higher peak intensity of the $a_{20}$ resonance in the sub-$a_1$ (and in the sub-$a_2$) phase assigns $a_{20}$ to the more mobile methyl resonance. In the sub-$a_2$ phase (Fig. 6D) only the arachidonoyl moiety methyl group has the mobility to give a high power proton decoupled peak.

Cross-polarization, Magic Angle Spinning $^{13}$C—The differential mobility of the stearoyl and the arachidonoyl moieties was further investigated by cross-polarization MAS NMR experiments, a kinetic process (49) where simultaneous irradiation of $^{13}$C and $^1$H resonances increases the carbon magnetization by polarization transfer from the protons. The subsequent depletion of the carbon and proton magnetization is caused by spin lattice relaxation processes, and the carbon relaxation rates depend on the carbon species (50). Thus, the carbon resonance intensities may not be directly representative of the relative numbers of carbon species in the sample (e.g., two methyl groups of the same relative number and with different signal intensities have differences in their spin lattice relaxation). This is due to differences in surroundings and mobility. Figs. 7-9 present cross-polarization, magic angle spinning spectra with contact times of 0.5 and 5.0 ms, as well as a high power
proton-decoupled spectrum of the CH$_2$ and CH$_3$ chemical shift regions.

These spectra of the $\alpha$, sub-$\alpha_1$, and sub-$\alpha_2$ phases are shown in Figs. 7–9, respectively. In Fig. 7 spectrum A is a high power proton-decoupled experiment and spectra B and C display cross-polarization experiments with contact times of 5.0 and 0.5 ms, respectively. The methyl resonances of the arachidonoyl and stearoyl moieties appear at different chemical shifts and are labeled a20 and s18, respectively, in the A panels of Figs. 7–9. Note the differential contact time dependences of the arachidonoyl and stearoyl moieties, shown in spectra B and C of these figures. In the solid sub-$\alpha_1$ phase (Fig. 8) spectrum A is a high power proton-decoupled experiment, and spectra B and C display cross-polarization experiments with contact times of 5.0 and 0.5 ms, respectively. The methyl resonances of the arachidonoyl and the stearoyl moieties appear at different chemical shifts, labeled a20 and s18, respectively (Fig. 8A). The differential contact time dependence of the arachidonoyl and stearoyl methyl groups is evident in spectra B and C. Furthermore, this contact time dependence in the sub-$\alpha_1$ phase is clearly different from the corresponding dependence in the $\alpha$ phase. The different contact time dependence of the stearoyl peak s(CH$_2$)$_n$ in the two solid phases $\alpha$ and sub-$\alpha_1$ is also displayed in Figs. 7 and 8. In both of these two phases ($\alpha$ and sub-$\alpha_1$) the stearoyl methyl group (s18) produces the larger methyl peak of the two methyl peaks, and the corresponding arachidonoyl methyl signal (a20) can be seen as a small shoulder on the upfield side of the stearoyl methyl signal. In Fig. 9, spectra A—C display CH$_3$ and CH$_2$ resonance region of SAG in the sub-$\alpha_2$ phase. Spectrum A is a high power proton-decoupled experiment, and spectra B and C display cross-polarization experiments with contact times of 5.0 and 0.5 ms, respectively. The methyl resonances of the arachidonoyl and stearoyl moieties appear as one broad peak in C, the spectrum acquired with the short contact time of 0.5 ms. In the spectrum of the longer contact time of 5.0 ms (spectrum B) and in the high power proton-decoupled spectrum (spectrum A), only the methyl group of the arachidonoyl moiety appears (spectrum A). The broad stearoyl moiety CH$_3$ resonance present in the experiment with 0.5-ms contact time (spectrum C) disappears when the contact time is increased to 5.0 ms, as well in the high power-decoupled experiment (spectrum A). The differences in spin lattice relaxation of the stearoyl and arachidonoyl methyl groups demonstrated by the different contact time dependences described above, as well as these methyl groups separate chemical shift values in the solid phases $\alpha$, sub-$\alpha_1$, and sub-$\alpha_2$, suggest that s18 and a20 reside in different environments, i.e., the stearoyl and arachidonoyl moieties do not pack beside each other.

Hydrated SAG—The NMR experiments on dry SAG (Figs. 3–9) were also carried out with hydrated SAG, and these are in excellent agreement with the corresponding results obtained on dry SAG. The hydrated phases have slightly better signal to noise ratio for some peaks, e.g., the three glycerol carbon peaks (g1, g2, and g3). This is probably due to increased mobility when hydrated.
DISCUSSION

The x-ray data of SAG shown here suggest a molecular organization that differs from that of a regular bilayer. In the regular bilayer arrangement SAG would be found in the conventional hairpin conformation, i.e. the stearoyl and arachidonyl chains would pack together and next to each other. The strong x-ray peak from a long spacing of \( \approx 62 \, \text{Å} \) in the molecular organization of SAG in the three solid phases would require that the SAG molecules extend about \( \approx 31 \, \text{Å} \) along the long axis of the unit cell. Even if the SAG molecule was parallel to the c axis and fully extended, it seems unlikely that it could be \( \approx 31 \, \text{Å} \) long. The 4 double bonds (\( \approx 1.27 \, \text{Å} \)) are shorter than the \( \text{CH}_2-\text{CH}_2 \) bonds, and this would shorten the extended chain. Computer models suggest that the SAG molecule in extended conformation is about \( \approx 26 \, \text{Å} \) long, and the corresponding bilayer composed of 26-Å long SAG molecules in a hairpin fashion would be \( \approx 52 \, \text{Å} \) thick. Thus, the x-ray data make unlikely a regular bilayer organization of SAG in the solid phases \( \alpha \), sub-\( \alpha_1 \), and sub-\( \alpha_2 \).

The NMR data demonstrate that in the \( \alpha \) phase the stearoyl moiety is in the solid crystalline phase (the main chain stearoyl \( \text{CH}_2 \) in resonance is found as a broad peak at 32.5 ppm in the \( \alpha \) phase and as an isotropic phase peak at 30.4 ppm). Furthermore, in the \( \alpha \) phase the arachidonyl moiety displays a mobility typical of the liquid crystalline phase. At the lower temperatures of the sub-\( \alpha_1 \) and sub-\( \alpha_2 \) phases, the solid phase character of the stearoyl carbon resonances and the higher mobility of arachidonoyl resonances persist, even though the arachidonoyl resonances show differences in mobility so that in the sub-\( \alpha_2 \) phase a20 to a10-a8 display mobility and give rise to high power proton-decoupled NMR peaks. In these spectra all stearoyl peaks have vanished due to the low mobility of these carbons. Of the glycerol resonances the one assigned to \( \text{g1} \) displays a line broadening that greatly exceeds the \( \text{g2} \) and \( \text{g3} \) resonances, when SAG is brought from the isotropic phase to the solid \( \alpha \) phase. This is in excellent agreement with the described behavior of the \( \text{sn-1} \)-bound stearoyl moiety. The highly different behaviors of the stearoyl and arachidonoyl moieties suggest segregation of these moieties when present in the solid phases \( \alpha \), sub-\( \alpha_1 \), and sub-\( \alpha_2 \). A V-shaped conformation of the SAG molecule with the stearoyl and arachidonoyl chains coming off the glycerol with an angle between their planes of about 115° achieves such a segregation of the stearoyl and arachidonoyl moieties and could explain the strong x-ray peak from a long spacing of 62–63 Å in the molecular organization. With this separation of the two acyl chains the stearoyls pack in a stearoyl layer and the arachidonoyls pack in an arachidonoyl layer. These two “monolayers” will stack onto neighboring stearoyl and arachidonoyl layers, without interdigitation, and presumably so that two stearoyl layers and two arachidonoyl layers meet and the separation of stearoyl “bilayers” (and the arachidonoyl bilayers) will be 62–63 Å (see Scheme 2). A V-shaped conformation of 94° has been found for \( \text{sn-1} \)-stearyl-3-oleylglycerol in an x-ray single crystal diffraction study (38), whereas the \( \text{sn-1} \)-stearyl-2-oleoylglycerol, on the other hand, gave rise to disordered crystal packing (37), probably due to disordered chain packing. In the \( \text{sn-1,2} \)-diacyl-
glycerol molecule in our study, the four double bonds of the arachidonoyl chain could make the two acyl chains sufficiently incompatible so that the two chains segregate when they pack in the solid phase. We emphasize, however, that only a definitive (single crystal) x-ray structure can resolve the uncertainties in the molecular organization of SAG in the solid phase.

For the biological implications, \( sn \)-1,2-SAG is formed rapidly from PIP2 in cell membranes during stimulation of cells by agonists that are coupled to PLC-\( \beta \) through G-proteins and to PLC-\( g \) through receptor tyrosine phosphorylation (51). This SAG is rapidly phosphorylated to \( sn \)-1-stearoyl-2-arachidonoyl phosphatidic acid by DAG kinase and thus exists only transiently in the membrane (22). However, the tendency of DAGs in general to form microdomains in membranes (30, 32) suggests that PLC-generated SAG also may become transiently concentrated in microdomains in the cell membranes. It is worth noting that many DAGs also may become transiently concentrated in microdomains in the cell membranes. It is worth noting that model organisms present in the solid phase (21) and in high power proton-decoupled spectrum only, the methyl group of the arachidonic moiety appear (A). The broad stearic moiety CH\(_3\) resonance present in the 0.5-ms contact time experiment (C) disappears when the contact time is increased to 5.0 ms as well in the high power proton-decoupled experiment (A).

![Diagram](image)

**Scheme 2.** Schematic representation of a possible conformation and packing of SAG in the \( \alpha \) phase. The model shows the immobile stearoyl and glycerol moieties as thick lines and the mobile arachidonoyl chains as thin lines. The packing arrangement shown with separate stearoyl bilayers and arachidonoyl bilayers is in accordance with the chain segregation indicated in the NMR data. The strong long spacing x-ray diffraction on 62.1 Å determines a stearoyl/arachidonyl interchain angle of \(-115^\circ\).
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for the various biological actions such as membrane destabilization, enzyme activation, and fusion events promoted by SAG or other 1-saturated/2-polyunsaturated glycerols (56).

Unsaturated DAGs are cone-shaped molecules with the hydroxyl apex at the interface. A few of these molecules can change a flat bilayer to a concave one. When the two chains are quite different, such as we have shown here for SAG, chain segregation may occur in the dry or nearly dry state. However, to obtain a chain segregated, extended conformation in a hydrated bilayer, the glycerol-OH and ester groups would either have to be buried in the center of the bilayer or one of the acyl chains would have to protrude into the aqueous layer. Both processes are thermodynamically unfavorable as it costs energy to bury –OH and ester groups in hydrocarbon or to force –CH₂ groups into water. A possible role for chain segregation in protein binding to membrane would be as outlined in Scheme 3. The formation of a few proximate SAG molecules in a membrane would cause a local cavity or dimple on the surface. An adjacent protein might hydrogen-bond to the SAG-OH(s) and perhaps to adjacent phospholipids on the rim of the dimple. If the protein had a sequestered hydrophobic tip that could specifically interact with the arachidonoyl chain(s), then chain segregation could occur with the protein interacting with the arachidonoyl chain in the dimple; the glycerol-OH bound to the tip of the protein, and the stearyl chain submerged deep in the hydrocarbon part of the bilayer. This speculation is reasonable, as the SAG-OH is hydrogen-bonded to protein and therefore can be submerged in a hydrophobic environment. The segregated arachidonoyl moiety interacts with the protein hydrophobic finger, allowing it to settle into the bilayer, and the stearyl group remains firmly anchored in the bilayer (Scheme 3).

Although highly speculative, the mechanism depicted in Scheme 3 takes into account the tendency of SAG to form the low energy, V-shaped structure suggested from the physicochemical results in the present work (Scheme 2). In an in vitro system Goldberg et al. (57) found that both SAG and sn-1,2-dioleoylglycerol (DO) gave pronounced activation of PKC, which seemed to be related to the increased tendency of SAG and DO to form non-bilayer lipid domains in ternary mixtures of saturated PC and PS (58), whereas other DAGs (dioleoylglycerol, dipalmitoylelglycerol, and oleoyl-acyl glycerol) gave little or no activation. Further studies by these authors (59) showed that SAG and DO also gave conformational changes in the phosphatidylcholine head groups that correlated with the DAG-induced activation of PKC. Studies by others (55, 56) with large unilamellar vesicles of saturated PC and PS also indicated that PKC activation may be related to the interphases between DO-rich and DO-poor domains in ternary mixtures with PC and PS.

We are unaware of physicochemical studies of neat DO similar to those we have performed with SAG, and we feel that it is unlikely that the acyl chains of a DAG with two identical acyls are able to segregate in the way we have shown here for neat SAG. However, since 1-stearoyl-2-oleoylglycerol packs poorly (37), it is possible that DO also packs anisotropically and, thus, may have a tendency to segregate and mix indiscriminately with other phospholipids accompanied by formation of DO-rich and DO-poor domains (56). The striking similarity between DO and SAG in physicochemical behavior and the ability to activate PKC (59, 60) support this view.

The tendency of SAG to attain a V-shaped conformation may represent a property of mixed chain DAGs in general which could provide a physicochemical basis for their fusogenic behavior (24, 28). Also, the non-lamellar and/or extended conformation with non-parallel acyls has been thought to promote attachment of proteins such as PKC to membranes (55, 56). It is also possible that SAG molecules produced during hydrolysis of PIP₂ by PLC exert tension on neighboring membrane molecules by the tendency of SAG to attain V-shape. If so, this would rearrange the local packing around the newly formed SAG molecules that may be part of the biological actions of SAG (Scheme 3).

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SCHEME 3. Possible mechanism for anchoring a PS-requiring protein to a biological membrane through formation of SAG. A, section of a bilayer is shown containing sn-1-stearoyl (thick lines)-2-arachidonoyl (semi-thick lines)-PIP₂ (dark gray) surrounded by PS molecules (light gray, each carrying a net negative charge on the head group) in one leaflet. B, same bilayer as in A after PI-specific PLC action. sn-1-Stearyl-2-arachidonoyl-PIP₂, has been converted to SAG (the glycerol moiety with the free OH is indicated by dark, small squares). Since SAG is a cone-shaped molecule, a dimple is made in the membrane with the acyl chains of the neighboring PS somewhat spayed. A membrane-anchoring domain of a protein is shown in juxtaposition to the dimple with positively charged amino acids positioned above the PS molecules and with a (extending) hydrophobic finger. C, the anchoring domain is fastened to the dimple through electrostatic attraction (arrows) between the PS heads and the positive charges in the anchoring domain and through hydrogen bonds (broken lines) between the tip of the finger protruding out of the anchoring domain and the hydroxyl group of SAG. D, the hydrophobic finger of the anchoring domain specifically interacts with the arachidonoyl chains of SAG which segregates from the stearyl chain, so that the entire SAG molecule is attaining the chain-segregated, low energy form depicted in Scheme 2, and the protein becomes firmly anchored.
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