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CRAC motif peptide of the HIV-1 gp41 protein thins SOPC membranes and interacts with cholesterol

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Abstract

This study uses low-angle (LAXS) and wide-angle (WAXS) X-ray synchrotron scattering, volume measurements and thin layer chromatography to determine the structure and interactions of SOPC, SOPC/cholesterol mixtures, SOPC/peptide and SOPC/cholesterol/peptide mixtures. *N-acetyl*-LWYIK-amide (LWYIK) represents the naturally-occurring CRAC motif segment in the pretransmembrane region of the gp41 protein of HIV-1, and *N-acetyl*-IWYIK-amide (IWYIK), an unnatural isomer, is used as a control. Both peptides thin the SOPC bilayer by ~3 Å, and cause the area/unit cell (peptide+SOPC) to increase by ~9 Å^2 from the area/lipid of SOPC at 30 °C (67.0±0.9 Å^2). Model fitting suggests that LWYIK's average position is slightly closer to the bilayer center than IWYIK's, and both peptides are just inside of the phosphate headgroup. Both peptides increase the wide-angle spacing *d* of SOPC without cholesterol, whereas with 50% cholesterol LWYIK increases *d* but IWYIK decreases *d*. TLC shows that LWYIK is more hydrophobic than IWYIK; this difference persists in peptide/SOPC 1:9 mole ratio mixtures. Both peptides counteract the chain ordering effect of cholesterol to roughly the same degree, and both decrease K_C, the bending modulus, thus increasing the SOPC membrane fluidity. Both peptides nucleate crystals of cholesterol, but the LWYIK-induced crystals are weaker and dissolve more easily.

Keywords: HIV-1; Biomembranes; Lipid bilayers; CRAC motif peptide; Cholesterol crystals; SOPC

1. Introduction

Domain formation in membranes has generated considerable interest since the discovery of partitioning of cholesterol into detergent-insoluble lipid-specific fractions during cell membrane isolation [1]. These domains, or "rafts", are thought to sequester proteins that perform a specific role, such as GPI-anchored signaling via second messengers. In the HIV-1 membrane, there are two proteolytic cleavage proteins of gp160 that may use lipid rafts: gp120, which recognizes and docks to the T-cell membrane, and gp41, which catalyzes the fusion step between the T-cell and the HIV membrane. A structural motif in the V3 loop of HIV-1 gp120 has a high affinity for both cholesterol and sphingomyelin [2]. In gp41 there is a highly-conserved consensus sequence having the pattern L/V-(X)(1–5)-Y-(X)(1–5)-R/K in which X(1–5) represents 1–5 residues of any amino acid. This sequence was first identified in the peripheral benzodiazapene receptor which may have a role in facilitating cholesterol transport into mitochondria [3]. This CRAC motif, or cholesterol recognition amino acid consensus sequence, is located adjacent to the transmembrane region of gp41. In HIV-1 this sequence is LWYIK, which has been shown to bind to cholesterol using cholesteryl-hemisuccinate agarose [4] and from studies using MAS/NMR and DSC [5]. HIV infection requires cholesterol in the HIV membrane [6] and mutations in the CRAC sequence reduce HIV infectivity [7]. In the present work, we use X-ray diffuse scattering to probe structure and material properties of SOPC membranes with the CRAC motif peptide LWYIK, or the non-CRAC motif isomer, IWYIK, in the absence and presence of cholesterol. These peptides are two of several with related

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sequences that were investigated by differential scanning calorimetry (DSC) [5,8], and differences were found in the abilities of these peptides to inhibit cholesterol/lipid interaction and to cause cholesterol crystallization. Addition of cholesterol to lipid membranes is well-known to progressively lower the enthalpy of the lipid's sharp main phase transition and to induce a broader melting component [9]. Addition of LWYIK, but not IWYIK, reversed this effect [8]. Both LWYIK and IWYIK caused crystals of cholesterol to form at a 9:1 SOPC/peptide mole ratio in 50 mol % SOPC/cholesterol mixtures, but the enthalpy of melting these crystals was almost two-fold higher in the IWYIK samples. This suggests that IWYIK binds to the SOPC thereby displacing cholesterol from its interaction with SOPC; cholesterol then crystallizes. By contrast, LWYIK may facilitate the segregation of cholesterol into cholesterol-rich domains by binding directly to cholesterol causing the sterol to surpass its solubility limit in the membrane.

Our recently developed low-angle X-ray diffuse scattering (LAXS) technique provides accurate structures of pure bilayers [10–13] and bilayer/cholesterol mixtures [14] in their fully hydrated, fluid (liquid-crystalline) state. This LAXS methodology provides the experimental form factors $F(q_z)$ of the bilayer, with and without peptide. These form factors are then fit to a model of the SOPC bilayer which provides structural information such as area and thickness of SOPC membranes with peptides. Diffuse LAXS data also provides the membrane bending modulus K_C that measures how much energy is required to bend the membrane ($E = 1/2\ K_C\ C^2$, where the curvature $C = R^{-1}$, and R is the radius of curvature). In this study we determine how LWYIK and IWYIK affect K_C of SOPC membranes in the presence and absence of cholesterol. Wide-angle X-ray scattering (WAXS) enables us to determine how chain orientational order and lateral chain packing is affected by cholesterol and peptides [15,16]. In addition, the issue of precipitation of cholesterol crystals is examined using X-ray diffraction. Thin layer chromatography reveals the difference in hydrophobicity of these peptides. Finally, volume measurements are used in the SOPC bilayer structure determination [17].

2. Materials and methods

2.1. Materials

1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (SOPC) (Lot 180-181PC-48) was purchased from Avanti Polar Lipids (Alabaster, AL) in lyophilized form and used without further purification. D_2O/H_2O mixtures were made with Barnstead nanopure water and deuterium oxide (99.9 atom % D) (Aldrich, Milwaukee, WI). HPLC grade chloroform was purchased from Aldrich. The peptides *N-acetyl*-LWYIK-amide (LWYIK or L) and *N-acetyl*-IWYIK-amide (IWYIK or I) were purchased from the Biomedical Research Support Facilities, Synthetic Peptide Application Lab, at the University of Pittsburgh. Their purity was checked at the Center for Molecular Analysis at Carnegie Mellon University using electrospray ionization mass spectrometry. Both were found to have the same molecular weight (763.4 Da) and a purity of >99%.

2.2. Thin layer chromatography (TLC)

TLC of lipids, peptides and peptide/lipid mixtures was carried out in order to discern any differences in the peptides, which had the same molecular weight, and also to determine if lipid and/or peptide breakdown occurred during X-ray exposure or during the experimental procedures. The protocol and results are described in Supplementary Material.

2.3. Volume measurements

Volume measurements of pure SOPC and SOPC/cholesterol mixtures were carried out as described in Greenwood et al., 2006 [18]. Mixtures of D_2O and H_2O were prepared at known densities. Chloroform was added to ~3 mg of dry SOPC or SOPC/cholesterol and then the solvent was evaporated using a stream of nitrogen and subsequent overnight drying in a fume hood. When peptides were used, they were first solubilized in methanol by vortexing and added to the lipids in chloroform before evaporation of solvent. 3 ml of the D_2O/H_2O mixture was added to the dried mixtures. The samples were hydrated by vortexing and thermal cycling three times between 50 °C and −20 °C. The samples were centrifuged at 1380 ×*g* in a Fisher Centrific 228 desktop centrifuge (Pittsburgh, PA) located within a home-built temperature controlled chamber which was maintained at 30 °C using a YSI Model 72 proportional temperature controller (Yellow Springs, OH). The lipid was observed to either sink or float, depending on its density relative to that of the D_2O/H_2O solution. Temperatures were accurate to ±0.5 °C and specific volumes were determined to an accuracy of ±0.0005 ml/g.

2.4. Light Scattering

The density centrifugation results for samples with peptides were difficult to read, often yielding both floating and sinking lipid in the same vial. Therefore, dynamic light scattering was carried out in order to determine the size of the multilamellar vesicles (MLVs) formed in the absence and presence of peptides and cholesterol. Dynamic light scattering (DLS) was carried out using a Malvern particle sizer (Worcestershire, UK) that was calibrated with a polystyrene bead standard of 8020 Å diameter. DLS measures Brownian motion and relates this to the size of the particles. 1.5 ml containing 0.5–1 mg MLVs, was loaded into a glass cuvette and the Brownian motion was monitored over 1 min, nine times in succession and the results were averaged.

2.5. X-ray sample preparation and scattering experiments

4 mg SOPC or SOPC/cholesterol was first dissolved in chloroform, and peptide in a methanol stock solution was added in a 1:9 mole ratio to total lipid. The solvent was removed using a KNF filtration pump (Trenton, NJ). For oriented samples 200 µl 1:1 chloroform/methanol was added to this mixture which was then vortexed and plated onto 30 × 15 × 1 mm silicon wafers using the rock and roll technique [19,20]. Samples were dried one day in a glove box and one day in a fume hood and trimmed. Hydration from water vapor was then carried out in a thick-walled hydration chamber [11] and continued until the lamellar *D*-spacing was within 1–2 angstroms of the *D*-spacing obtained with excess water in X-ray capillaries (*vide infra*). Oriented X-ray data were taken at the Cornell High Energy Synchrotron Source (CHESS) using the G1 station on two separate trips with wavelength 1.2742 or 1.1797±0.012 Å. The beam was .2Hx.6Vmm or .2Hx.2V mm and the total beam intensity was 10^{11} photons/sec. The sample was ~10 µm thick along the normal to the ~2000 bilayers and its dimension along the direction of the beam was either narrow (5 mm) or wide (13 mm) for low-angle X-ray scattering (LAXS) on two separate trips, and narrow (4 mm) for wide-angle X-ray scattering (WAXS) measurements. The flat samples were rotated from −3 to 7 degrees in θ relative to the beam during the 30–60 s LAXS exposure and were X-rayed at fixed θ for the 10–20 s WAXS exposure. For WAXS, $\theta = 0.2°$ was used to first collect lipid scattering and then $\theta = -0.2°$ was used to collect background chamber scattering that was subtracted from the lipid data. Data were collected using a Flicam CCD (Finger Lakes Instrumentation, Lima, NY) with a 1024×1024 pixel array and pixel size 0.06978 mm/pixel. The sample-to-CCD distance was 400 mm for LAXS and 155 mm for WAXS.

Fully hydrated *D*-spacings of samples in excess water were prepared by weighing 1 mg of dry sample thoroughly mixed (*vide supra*) with 40 µl milli-Q water into small nalgene vials. These were vortexed and thermally cycled three times between 50 °C and −20 °C and loaded into glass X-ray capillaries. These MLV samples were X-rayed at 30 °C using a Rigaku RUH3R microfocus