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Structure of Dehydroergosterol Monohydrate and Interaction with Sterol Carrier Protein-2

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Abstract

Dehydroergosterol [ergosta-5,7,9(11),22-tetraen-3 β -ol] is a naturally-occurring, fluorescent sterol utilized extensively to probe membrane cholesterol distribution, cholesterol-protein interactions, and intracellular cholesterol transport both *in vitro* and *in vivo*. In aqueous solutions, the low solubility of dehydroergosterol results in the formation of monohydrate crystals similar to cholesterol. Low temperature x-ray diffraction analysis reveals that dehydroergosterol monohydrate crystallizes in the space group P2₁ with 4 molecules in the unit cell and monoclinic crystal parameters $a = 9.975(1)\text{\AA}$, $b = 7.4731(9)\text{\AA}$, $c = 34.054(4)\text{\AA}$, and $\beta = 92.970(2)^\circ$ somewhat similar to ergosterol monohydrate. The molecular arrangement is in a slightly closer packed bilayer structure resembling cholesterol monohydrate. Since dehydroergosterol fluorescence emission undergoes a quantum yield enhancement and red-shift of its maximum wavelength when crystallized, formation or disruption of microcrystals was monitored with high sensitivity using cuvette-based spectroscopy and multi-photon laser scanning imaging microscopy (MPLSM). This manuscript reports on the dynamical effect of sterol carrier protein-2 (SCP-2) interacting between aqueous dispersions of dehydroergosterol monohydrate microcrystal donors and acceptors consisting not only of model membranes but also vesicles derived from plasma membranes isolated by biochemical fractionation and affinity purification from Madin-Darby canine kidney cells. Furthermore, this study provides real-time measurements of the effect of increased SCP-2 levels on the rate of disappearance of dehydroergosterol microcrystals in living cells.

Keywords

ergosterol; dehydroergosterol; fluorescent sterols; crystals

INTRODUCTION

Cholesterol is required for proper structure and function of multiple transport and signaling pathways in mammalian membranes, especially plasma membranes (rev. in (1-9)). Furthermore, cholesterol is also a required substrate for peroxisomal enzymes involved in the synthesis of bile acids (for intestinal lipid absorption) and for the synthesis of steroids—potent

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hormones that regulate glucose metabolism, lipid metabolism, reproduction, etc. Conversely, accumulation of excess cholesterol results in formation of cholesterol crystals in membranes (rev. in (10-12) and lysosomes (rev. in (13-15)). Accumulation of excess cholesterol in the plasma membranes inhibits numerous intracellular functions (mitochondrial ATP-ADP exchange, microsomal UDP-glucuronyltransferase, Na^+ - Ca^{++} exchange in heart sarcoplasmic reticulum) but also plasma membrane functions such as ion transport (Na^+ , K^+ -ATPase, anion transport), receptor function (acetylcholine receptor, insulin receptor), nutrient transport (glucose), proteins involved in transmembrane signaling (adenylate cyclase), and others (rev. in (13,16,17)). Formation of intracellular cholesterol crystals is cytotoxic (rev. in (13,18) and can result in formation of atherosclerotic plaques (19-26). It is thought that excess cholesterol-induced death of macrophages is the main cell type that undergoes necrosis near the lipid cores of atherosclerotic lesions (rev. (13)). Thus, excess cholesterol is associated with significant pathologies including atherosclerosis, cardiovascular disease, Niemann-Pick C disease, Alzheimer's disease, as well as others.

While enhanced free cholesterol efflux, increased esterification, and decreased cholesteryl ester droplet hydrolysis have been considered as serving protective roles against cholesterol-induced macrophage cytotoxicity (rev. in (13)), some early studies suggest a potential role for intracellular sterol binding/carrier proteins such as sterol carrier protein-2 (SCP-2). SCP-2 has been detected in macrophages and arteries (27,28). Furthermore, incubation of rat peritoneal macrophages with acetylated LDL (AcLDL) resulted in a dose- and time-dependent increase not only in cellular cholesterol, but also in both SCP-2 protein and mRNA which were upregulated as much as 2.6-fold (15,29). In contrast, hepatic SCP-2 and SCP-x (product of alternate transcription site of the SCP-2 gene) were both deficient in adult Niemann-Pick C mice, a murine model sharing similar pathologies and lysosomal cholesterol accumulation as observed in human NPC disease (30,31). Finally, hepatic SCP-2 protein levels were reduced 60% in mildly and 90% in severely hypercholesterolemic diabetic rats (32). Taken together, these data suggest a potential protective role for SCP-2 in accumulation of crystalline cholesterol and cytotoxicity. However, the mechanism(s) whereby SCP-2 may be protective is as yet unclear. However, both *in vitro* and intact cell studies indicate that SCP-2 dramatically enhances cholesterol transport from a variety of model and biological membranes, including lysosomal membranes (rev. in (1-3,33,34)). While these studies suggest that SCP-2 similarly facilitate removal of cholesterol from cholesterol crystals, this possibility remains to be examined.

Because cholesterol is not readily visualized in real-time either *in vitro* or in living cells, the availability of a fluorescent sterol with properties closely mimicking those of cholesterol represents a major technological advance for elucidating mechanism(s) of protein-mediated sterol crystal dissolution, sterol organization in membranes, sterol dynamics, and sterol-protein interactions. Since the discovery of dehydroergosterol (DHE) in membranes of eukaryotes such as yeast *Candida tropicalis* (35) and Red Sea sponge *Biemna fortis* (36), DHE has been studied in model membranes (rev. in (37-39,39,40)), biological membranes of various intracellular organelles including plasma membrane sub-fractions (rev. in (1-3,9,34,41,42,42,43)), lipoproteins (rev. in (44-47)), cholesterol binding protein-sterol complexes (rev. in (48-50)), and annular cholesterol surrounding hormone receptors (51,52). These studies have shown DHE to be an excellent probe molecule with structural and functional properties that closely mimic those of cholesterol. More recent studies involve fluorescence resonance energy transfer (FRET) from DHE (3,41,53) to other sterols or to protein aromatic amino acid residues to determine intermolecular interaction distances (48,54-56). DHE has provided direct visualization of sterol in living cells by early confocal (41), multi-photon (41), and video (57) studies. Three-photon excitation laser scanning microscopy has enhanced real-time visualization of DHE in sterol-rich and —poor domains in the plasma membrane of living cells

(38,58,59) while improved video imaging allowed resolution of DHE in larger structures such as microvilli/filopodia in living cells (60,61).

Because of these properties of DHE and the propensity of DHE to form microcrystals in aqueous buffers (39,48,54) at very similar concentrations as cholesterol (19,20,62,63), DHE was used in the present investigation to: (i) elucidate the crystal structure of DHE monohydrate, (ii) examine spontaneous transfer from DHE microcrystals, (iii) determine if SCP-2 enhances DHE transfer from DHE microcrystals to acceptor cholesterol crystals, model membranes, plasma membranes, and sub-fractionated vesicles, (iv) examine the real-time intracellular effects of SCP-2 on microcrystalline DHE within living cells by MPLSM.

MATERIALS AND METHODS

Materials

Ergosterol (Fig. 1A) was used to synthesize dehydroergosterol (Fig. 1B) as described previously (38,64). The ergosterol (98% pure) was purchased from Steraloids (Wilmington, NH). Materials used in the synthesis included Burdick-Jackson (Muskegon, MI) "Purified Plus" solvents (methanol, chloroform, ethyl ether) and JT Baker (Mallinckrodt Baker, Phillipsburg, NJ) "Baker Analyzed" glacial acetic acid (99+% pure), acetic anhydride (99+% pure) from VWR (Atlanta, GA), and anhydrous mercuric acetate from Fisher Scientific (Pittsburgh, PA) in order to obtain the highest purity of DHE (>99%) determined as described (65,66). Human recombinant 13.2 kDa sterol carrier protein-2 (SCP-2) was isolated and purified as described earlier (67). Lipids including 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1,2-dioleoyl-*sn*-glycero-3-[phospho-L-serine] (DOPS), and cholesterol were purchased from Avanti Polar Lipids (Alabaster, AL).

Crystal Growth

Colorless needle shaped crystals of DHE were grown from slow evaporation of a saturated 95% ethanol solution. A Bausch and Lomb 10x microscope was used to identify a suitable colorless needle 0.2mm × 0.2mm × 0.05mm from a representative sample of crystals of the same habit. The crystal was coated in a cryogenic protectant (mineral oil), and was then fixed to a glass fiber which in turn was fashioned to a copper mounting pin. The mounted crystal was then placed in a cold nitrogen stream (Oxford) maintained at 110K.

Crystallography

A BRUKER SMART 1000 X-ray three-circle diffractometer was employed for crystal screening, unit cell determination, and data collection (Bruker-AXS, Madison, WI). The goniometer was controlled using the SMART software suite, version 5.056 (Microsoft NT operating system). The sample was optically centered with the aid of a video camera such that no translations were observed as the crystal was rotated through all positions. The detector was set at 5.0 cm from the crystal sample (CCD-PXL-KAF2, SMART 1000, 512×512 pixel). The X-ray radiation employed was generated from a Mo sealed X-ray tube ($K_{\alpha} = 0.70173\text{\AA}$ with a potential of 50 kV and a current of 40 mA) and filtered with a graphite monochromator in the parallel mode (175 mm collimator with 0.5 mm pinholes).

Dark currents were obtained for the appropriate exposure time 30 sec and a rotation exposure was taken to determine crystal quality and the X-ray beam intersection with the detector. The beam intersection coordinates were compared to the configured coordinates and changes were made accordingly. The rotation exposure indicated acceptable crystal quality and the unit cell determination was undertaken. Sixty data frames were taken at widths of 0.3° with an exposure time of 30 seconds. Over 200 reflections were centered and their positions were determined. These reflections were used in the auto-indexing procedure to determine the unit cell. A suitable

cell was found and refined by nonlinear least squares and Bravais lattice procedures (Table 1). The unit cell was verified by examination of the *hkl* overlays on several frames of data, including zone photographs. No super-cell or erroneous reflections were observed. After careful examination of the unit cell, a standard data collection procedure was initiated. This procedure consists of collection of one hemisphere of data collected using omega scans, involving the collection 1201 0.3° frames at fixed angles for ϕ , 2θ , and κ ($2\theta = -28^\circ$, $\kappa = 54.73^\circ$), while varying omega. Each frame was exposed for 30 sec and contrasted against a 30 sec. dark current exposure. The total data collection was performed for duration of approximately 13 h at 110 K. No significant intensity fluctuations of equivalent reflections were observed.

Analysis of crystal structure

The structure of dehydroergosterol was solved by standard direct methods and further refined employing SHELXS and SHELX97(68). CCDC 639255 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from the Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif. Since chirality is known, reflections were merged since the configuration would be determined. In this file, the hydrogen atoms of the water molecules were disordered and therefore omitted. A large ratio of U_{eqmax}/U_{eqmin} was due to terminal CH_3 groups that may be dynamically disordered and as such was not modeled.

Dehydroergosterol Donors: Microcrystals in aqueous buffer

DHE microcrystal dispersions were formed by adding small amounts of DHE from ethanolic stock solutions to a buffered solution of PIPES, pH 7.4 as described earlier (38,66). No filtering was performed and the microcrystals were observed previously to exist in irregular shapes with sizes ranging up to several microns when created by this technique (38,54).

Cholesterol acceptors: Microcrystals in aqueous buffer

Cholesterol acceptor microcrystal dispersions were formed by adding small amounts of cholesterol from ethanolic stock solutions to a buffered solution of PIPES, pH 7.4 similarly as described for DHE earlier (38,66). The resultant solution of microcrystals was not filtered.

Membrane acceptors: large unilamellar vesicles

Large unilamellar vesicles composed of POPC: DOPS: cholesterol (55:10:30 molar ratio) were formed exactly as described earlier (38,69,70).

Membrane acceptors: plasma membrane, caveolin-1 and cholesterol-rich sub-fraction (CAVCR), and non-caveolin-1 and cholesterol-poor sub-fraction(non-CAVCP)

Madin-Darby canine kidney (MDCK, American Type Culture Collection) cells were grown as described previously(71) for 72 h at 37 °C and 5% CO₂ in the presence of Dulbecco's Modification of Eagle's medium (D-MEM) (Cellgrow/Mediatech, Herndon, VA). The high glucose medium was supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 0.25 µg/L Fungizone, 100 units/L penicillin, 100 µg/L streptomycin, and 5% serum supreme from (Bio-Whittaker/Canbex, East Rutherford, NJ) as well as 0.1 mM nonessential amino acids (Cellgrow/Mediatech, Herndon, VA), and 43.9 mM sodium bicarbonate (Invitrogen, Carlsbad, CA). Following washing with phosphate buffered saline (PBS), the cells were covered with serum-free medium and incubated with DHE (20µg/ml) added directly to the media from a stock solution derived from absolute ethanol and containing < 1 mol% butylated hydroxytoluene (BHT) that was originally stored at -80 °C. The amount of ethanol added was kept below 0.3% to prevent any deleterious effects on the cells. CAVCR (binding fraction) and non-CAVCP (non-binding fraction) were resolved by concanavalin-A affinity chromatography

from purified plasma membranes (42,43,71-73). Purity of the plasma membrane, binding, and non-binding fractions was determined as described in the cited papers using western blotting of a panel of protein markers for plasma membranes ($\text{Na}^+\text{K}^+\text{-ATPase}$, SRB1), caveolae (caveolin-1 and GM1), and non-caveolae ($\text{Na}^+\text{K}^+\text{-ATPase}$).

Excitation and emission anisotropy of DHE

Measurements of the wavelength dependence of anisotropy for both the excitation and emission of DHE were performed using a Cary Eclipse spectrofluorometer (Varian, Palo Alto, CA) with a Peltier temperature controlled holder kept at 24 °C. The slits of both the excitation and emission monochromators were set to 5 nm bandwidths with scan speed set at 120 nm/min. The emission scan of anisotropy was performed with an excitation wavelength of 324 nm. Two excitation scans of anisotropy were made with the fluorescence emission collected at 375 nm and 425 nm.

Measurement of DHE fluorescence anisotropy during exchange assays

All measurements of fluorescence anisotropy over time (~4 h) were made using a T-format of an ISS PC1 spectrofluorometer (ISS Instruments, Champaign, IL) with rotating cuvette turrets as well as with UV-grade Glan-Thompson excitation and emission polarizers. The excitation wavelength was set at 324 nm and the emission was collected using a Schott KV389 cutoff filter (Schott Optics, Duryea, PA). Baseline DHE fluorescence anisotropy of the donor microcrystals (2.5 μM 10 mM PIPES buffer, pH = 7.4) was measured for 20 min to ensure a stable baseline and obtain initial DHE anisotropy. After addition of 10-fold excess of acceptor (contains no DHE), DHE anisotropy was recorded at 20 s intervals for 4 h to monitor sterol transfer between DHE microcrystal donors and acceptor cholesterol microcrystals or membranes. Residual light scatter contributions were corrected using controls and subtracting the residual fluorescence anisotropy from all experimental data. To prevent inner filter artifacts, absorbance of sample solutions at the excitation wavelengths were <0.15. The equations

$r = \frac{I_{pl} - I_{pd}}{I_{pl} + 2I_{pd}}$ was where r is the anisotropy, I_{pl} and I_{pd} is the emission intensity measure with polarizers parallel and perpendicular, respectively, to the excitation polarizer set vertically, and I_T is total emission intensity (74).

Determination of sterol exchange: Polarization assay

Sterol exchange between DHE microcrystal donors and acceptor cholesterol microcrystals, large unilamellar vesicles, plasma membranes, binding fraction, or non-binding fractions was determined by using a fluorescent sterol (DHE) exchange assay as previously established (42,43,71,75). Time dependent anisotropy changes was modeled with the equation

$$r(t) = f_d(t) r_d(t) + f_a(t) r_a(t) \quad \text{Eq.(1)}$$

where $r(t)$ was the measured anisotropy over time, $f_d(t)$ was the fractional intensity resulting from the donor, $f_a(t)$ was the fractional intensity resulting from DHE transfer into the acceptor, $r_d(t)$ was the donor anisotropy, and $r_a(t)$ was the acceptor anisotropy for the membrane. Crystalline donor anisotropy ($r_d = 0.09$) was constant for microcrystalline DHE and approximately independent of the concentration of crystals. DHE crystalline anisotropy was highly depolarized as compared to the anisotropy of DHE within membrane vesicles, which varied depending upon concentration as a result of self-quenching effects as seen in other studies (76-79). The acceptor anisotropy was typically derived from the a standard curve that must be measured from varying dehydroergosterol mole fractions ($X_a = C_a/C_T$) of the particular acceptor and fit to the equation

$$r_a(X_a) = r_0 + b_1 * X_a + b_2 * (X_a)^2 \quad \text{Eq.(2)}$$

where r_0 is the anisotropy at infinite dilution(71). However, since for 10 fold dilution $0 < X_a < 0.1$ and $\frac{dr}{dX_a} \sim 0$, the effect of self-quenching would be minimal so that $r_a(t)$ was fixed to r_0 of each acceptor membrane type.

The fractional intensity contribution to the anisotropy of the DHE in the acceptor was described by the following equation:

$$f_a(t) = f_1(1 - e^{-at}) + f_2(1 - e^{-bt}) \quad \text{Eq.(3)}$$

where f_1 and f_2 are related to the fractional pool sizes of easily exchangeable cholesterol within the acceptor membrane vesicle with half-times

$$t_{1/2}^1 = \frac{\ln(2)}{a} \quad \text{and} \quad t_{1/2}^2 = \frac{\ln(2)}{b} \quad \text{Eq.(4)}$$

The donor fractional intensity is related to the acceptor contribution by the following equation:

$$f_d(t) = 1 - f_a(t) \quad \text{Eq.(5)}$$

Combining these concepts formulated the equation, $r(t) = (1 - f_a(t))r_d + f_a(t)r_a$ which is further simplified:

$$r(t) = r_d + [f_1(1 - e^{-at}) + f_2(1 - e^{-bt})](r_a - r_d) \quad \text{Eq.(6)}$$

The anisotropy curves were plotted and fitted by non-linear regression to Equation 5 using Sigma Plot 8.0 (Systat Software, San Jose, CA) and the half-times were calculated using Equation 3. The initial rates of the exchange of sterol from the microcrystalline form of DHE to membrane vesicles was calculated from the derivative of $r(t)$ evaluated at $t = 0$.

$$\frac{dr(t)}{dt}|_{t=0} = [f_1a + f_2b](r_a - r_d) \quad \text{Eq.(7)}$$

DHE spectra at the beginning and at the end of exchange assays

Steady state fluorescence emission spectra of DHE excited at 324 nm were obtained with a PC1 Photon Counting Fluorometer (ISS Instr., Champaign, IL). Light scatter was minimized by use of narrow excitation slits, low concentration, and cutoff filters. Residual Raman scattering was subtracted. Absorbance at the excitation wavelength was maintained below 0.15 to avoid potential inner filter effects. Spectra were analyzed with Grams32 (Thermo Galactic, Salem, NH).

Cell culture of control and SCP-2 overexpressing L-cells

Murine L cells and L-cells transfected with cDNA corresponding to the 15-kDa pro-SCP-2 protein were cultured on Lab-Tek Chambered coverglass (Nunc, Naperville, IL) at 37 °C and 5% CO₂ overnight in Higuchi medium (80), supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), as described earlier (42). The cells with transfected cDNA overexpressed SCP-2 protein so that it comprised 0.036% of the total cytosolic proteins while the level in the control mock transfected L-cells was negligible. Subsequently, the cells were washed with PBS followed by the addition of serum-free medium containing DHE (20 µg/ml) added directly from the ethanolic stock solution previously described (<0.3% ethanol added). The cells on the cover glass was incubated for 24 h and washed with PBS followed by the addition of serum containing media. Cells were removed for imaging at approximately 12 h time points.

Multi-photon laser scanning microscopy (MPSLM) of dehydroergosterol

A Bio-Rad MRC-1024 MP (Zeiss, Thornwood, NY) laser scanning confocal/multi-photon microscopic imaging system was used to obtain images of the fluorescence emission of the structural distribution of DHE within living cells over the 60 h time period. The MRC-1024 MP was coupled to a Zeiss Axiovert 135 inverted microscope (Zeiss, Thornwood, NY) with heated incubator and stage. A Zeiss 40x Plan-Apochromat (1.4 N.A.) was used for real-time imaging from $t = 0$ to $t = 60$ hrs. The cells on chambered cover glass were maintained in a 37 °C CO₂ incubator between imaging time points. Imaging software, MetaMorph 4.5 (Molecular Devices, Downingtown, PA), was used to determine the mean integrated intensities from labeled cells (both control and SCP-2 overexpressing L-cells) at each time point from collective measurements of ~50 cells while mean integrated intensities of autofluorescence were obtained similarly using unlabeled cells (both control and SCP-2 overexpressing L-cells). Multi-photon excitation of the DHE was provided by a pulsed mode-locked Coherent Mira 900F (Coherent, Palo Alto, CA) tuned to 900 nm. Two photomultipliers of an external detector system (Dr. Zipfel, Cornell University, Ithaca, NY) was used in combination with two emission filters (Chroma Technology, Rockingham, VT), 375/50 nm and 455/30 nm, to simultaneously collect the emission in two distinct spectral regions. The ratio of the integrated measurements collected from the two channels was used to determine the intracellular monomeric vs. crystalline distributions of DHE.

Data and Statistical Analyses—Curve-fitting and non-linear regression analyses were performed within the scientific data and analysis and graphing software, SigmaPlot v.8 (Systat Software, Inc., San Jose, CA).

RESULTS

Dehydroergosterol monohydrate crystal structure solution and refinement

DHE monohydrate crystal screening, unit cell determination, and data collection on a suitable cell were performed as described in Methods. The data was reduced (SAINT, Bruker-AXS, Madison, WI.) and merged. The structure of DHE was solved by standard direct methods and further refined using software developed by G.M. Sheldrick as described in Methods. The crystal refinement results were provided in Table 2.

A dehydroergosterol monohydrate crystal was grown from saturated 95% ethanol solution to the dimensions $0.20 \times 0.20 \times 0.10$ mm³ along the a , b , c axes. The dehydroergosterol monohydrate symmetry of diffraction was found to be monoclinic [Note: the cholesterol monohydrate has an apparent monoclinic symmetry, but close inspection of the twinned crystals revealed triclinic symmetry; (81,82)]. The space group was thus $P2_1$, with reduced cell parameters $a = 9.9747(11)$ Å, $b = 7.4731(9)$ Å, $c = 34.054(4)$ Å, $\alpha = 90^\circ$, $\beta = 92.970^\circ(2)$, $\gamma = 90^\circ$. The DHE unit cell is smaller than that reported by Bernal (83) who reported an A2 structure of 8 molecules in the unit cell with $a = 9.5$ Å, $b = 7.5$ Å, $c = 75.2$ Å, $\beta = 96^\circ$ from data recorded with photographic film from an impure crystal. In this case the cell reported by Bernal in 1940 was a superstructure of the cell reported in this manuscript. The volume of the dehydroergosterol monohydrate crystal unit cell was $2535.1(5)$ Å³, with a density of 1.081 mg/m³, in agreement with that calculated from the assuming the unit cell contained $4(C_{28}H_{44}O_2) \cdot 4H_2O$. A dimer unit of two $(C_{28}H_{44}O_2) \cdot H_2O$ forms the asymmetric volume of the unit cell with 4 molecules of $(C_{28}H_{44}O_2) \cdot H_2O$ in the unit cell (Fig. 2A,B) similar to ergosterol monohydrate, while cholesterol monohydrate has 8 molecules in its unit cell. From the 32,269 reflections collected, only 8865 with intensity $I > 2\sigma(I)$ were obtained and used for resolving the structure. Large amplitudes of atomic thermal vibrations primarily accounted for the high proportion of weak reflections. All 64 [only asymmetric volume atoms are detected, the remainder are generated by symmetry] n-hydrogen atoms were detected in electron density

maps. A full-matrix least-squares on F^2 revealed goodness of fit of 1.041, final R indices [$I > 2\sigma(I)$] of $R1=0.0688$ and $wR2=0.1766$, and R indices (all data) $R1=0.0688$ and $wR2=0.1766$.

The molecules are packed in staggered layers (Fig. 2A,B) with the intermolecular separation of 5.50(1) Å between the centroids of the molecules. The hydrophilic ends of the DHE molecules are located inward toward the water molecules while the hydrophobic ends of the molecules are directed outward toward the hydrophobic ends of the adjacent DHE molecules. The hydrogen atoms apart of the water molecules were disordered and have not been included (Fig. 2A,B). The ring system of the DHE molecule (Fig. 1B) is approximately planar where the ring A had a chair conformation, ring B was significantly planar, ring C was formed a partial chair structure with that was planar except for the last carbon joining ring D, and ring D was a distorted. The DHE molecules stack in a parallel mode normal to the diagonal of the *a* and *c* axes.

The effect of excitation and emission wavelength on anisotropy of aqueous dispersions of microcrystalline dehydroergosterol

Anisotropy excitation spectra were acquired as described in the Methods and graphed as shown in Fig. 3A. The open circles represent the excitation anisotropy spectrum acquired with the emission monochromator fixed to 375 nm with a 5 nm bandwidth. This spectrum revealed that the anisotropy was relatively constant across much of the excitation wavelengths with only a slight increase in anisotropy at the longer wavelengths. Typically this observation has been made when transition moments are near collinear which occurs when the emission originates from the same state as the absorption band. The closed circles (Fig. 3A) represent the excitation anisotropy spectrum acquired with the emission monochromator fixed to 425 nm with a 5 nm bandwidth wherein the greatest enhancement in emission arises under crystal formation. In this spectrum, the anisotropy was relatively constant across the shortest wavelengths but decreased gradually at the longer wavelengths indicative of a larger displacement of transition moments. Due to the restrictive environment within the crystal and the observed higher quantum yield, this was suggestive of an involvement in a different state possibly the result of formation of an excited state dimer rather than simple homotransfer or self-quenching that has been observed in vesicles (38,54). Despite the different states involved in the fluorescence emission, the emission anisotropy spectrum (Fig. 3B), acquired with excitation wavelength fixed to 324 nm and a 5 nm bandwidth, showed very little dependence upon wavelength. This was in agreement with the excitation anisotropy measured at 324 nm (Fig. 3A) where only a slight difference existed using the acquisition emission wavelengths of 375 nm and 425 nm.

Sterol exchange between donors and acceptors

DHE has proven to be a useful probe for cholesterol transfer because it is a naturally-occurring fluorescent sterol (35,36) exhibiting structural and functional properties closely resembling those of cholesterol as detailed in the Introduction (rev. in (1,2,38,39,45,51,75,84-87)). The theoretical basis for the DHE exchange assay has been previously established with respect for exchange of sterol from donor membranes to acceptor membranes (42,43,71,76,77,88,89). Briefly, at high concentrations of DHE in membranes, significant depolarization or decrease in anisotropy occurs as a result of resonance energy transfer (RET) between DHE molecules. Upon addition of 10-fold excess (by sterol) acceptor cholesterol microcrystals or acceptor membranes, the donor DHE microcrystal exchanges DHE one-for-one with cholesterol in the acceptor, resulting in release of DHE from self-quenching, thereby increasing polarization or anisotropy. Typically, a standard curve is determined by measuring the polarization or anisotropy as a function of the mole fraction of the fluorescent sterol within donor and acceptor (if different membrane type). Thus, a determination is made from the standard curve of the mol percent of DHE that has been exchanged. However, in this study, the donor consists of

microcrystalline DHE with low anisotropy ($r \sim 0.09$) that remains constant until the amount of aqueous DHE falls below the critical micelle concentration (CMC) which is quite low (~ 30 nM). By implication, sterol exchange observed by increasing anisotropy would be the result of increasing localization of sterol within the acceptor. Because of the ten-fold excess of acceptors, the acceptor anisotropy occurs between $r(X_a = 0) = r_0$ (anisotropy at infinite dilution) to $r(X_a = 0.1) \approx r_0$. This approximation implies that only the fractional contributions of the anisotropy depend upon the sterol mole fractions (see Methods).

Effect of cholesterol transfer proteins on sterol exchange between crystalline dehydroergosterol and crystalline cholesterol

Sterol exchange between dehydroergosterol microcrystals and cholesterol microcrystals was determined with a fluorescence polarization assays as described in Methods. Baseline anisotropy of DHE microcrystals in buffer alone was essentially unchanged over a 4 h time period (Fig. 4A). At the end of this incubation period, the emission spectrum of DHE microcrystals (excited at 324 nm) was obtained. As shown in Fig. 4B, the raw emission spectra exhibited 4 major peaks near 355, 373, 397, and 426 nm. After correction for background, the ratios of emission peaks as 426 nm/355 nm and 426 nm/373 nm were 3.7 and 2.5, respectively—consistent with DHE being localized primarily in crystalline structures as described earlier (38,48,54). The ratio of 455 nm/375 nm was 1.5 at the endpoint.

To examine spontaneous sterol exchange between donor and acceptor microcrystals, tenfold excess acceptor cholesterol microcrystals were added to donor DHE microcrystals in aqueous buffer. The DHE anisotropy increased slowly (Fig. 4C), consistent with one-for-one exchange of DHE in the donor DHE microcrystals and cholesterol in the cholesterol microcrystal acceptors. Analysis as described in Methods revealed that the anisotropy change best fit a single component with a long half-time $t^{1/2}_1 = 130$ min and a very low exchangeable fraction of 0.057 of the total donor sterol (Table 3). After correction for background, the ratio of 455 nm/375 nm was 1.4—again consistent with DHE being localized primarily in crystalline environment. These findings suggested that very little DHE had transferred to the cholesterol microcrystal and the environment basically exhibited similar properties as that in DHE microcrystals determined by spectral analysis.

To determine if proteins known to enhance lipid transfer between membranes might also facilitate DHE exchange between microcrystals, the effects of two classic cholesterol transfer proteins were tested: (i) An extracellular cholesterol transfer protein (bovine serum albumin, BSA); (ii) An intracellular cholesterol transfer protein (sterol carrier protein-2, SCP-2). Addition of albumin or SCP-2 to DHE microcrystal donors alone did not significantly alter the anisotropy or spectral properties (not shown). Addition of bovine serum albumin to DHE microcrystal donors in the presence of ten-fold excess cholesterol microcrystal acceptors (Fig. 4E) significantly decreased the half-time of transfer (Table 3). The initial rate did not change significantly and the overall exchangeable fraction, though slightly lower, was similar to the spontaneous (Table 3) as corroborated by the spectral properties (Fig. 4F) where the ratio of 455 nm/375 nm was 1.3. In contrast, SCP-2 rapidly increased the DHE anisotropy with increasing time of DHE exchange between DHE microcrystal donors and cholesterol microcrystal acceptors (Fig. 4G). Analysis of the exchange curves as described in Methods indicated that they optimally fit two components: (i) the contribution $t^{1/2}_1 = 74 \pm 4$ min with a total exchangeable fraction of 0.0867 ± 0.0003 and (ii) the contribution $t^{1/2}_2 = 6.1 \pm 0.3$ min with a total exchangeable fraction of 0.0732 ± 0.0006 . The first fraction was increased only about 1.5-fold more than the spontaneous (Table 3), but the $2.5 \mu\text{M}$ SCP-2 significantly reduced the half-time $t^{1/2}_1$ of DHE exchange by 2.2-fold which was similar to the $2.5 \mu\text{M}$ BSA (Table 3). Furthermore, SCP-2 induced the formation of a second component, nearly as large as the first. The half-time $t^{1/2}_2$ of the new component induced by SCP-2 was very short, near 6 min

or a nearly 22-fold faster exchange (Table 3) which contributed to an initial rate increase of 29-fold over the spontaneous. Finally, SCP-2 significantly altered the emission spectra of DHE at the end of the exchange process (Fig. 4H). After correction for background, the ratio of 455 nm/375 nm was 0.6—consistent with a significant amount of transferred DHE being localized in a less crystalline/more disordered region of the acceptor cholesterol microcrystals.

In summary, spontaneous DHE exchange between DHE microcrystals and cholesterol microcrystals was best fit by a single, very slow component. While BSA only decreased the half-time of the slow component of DHE exchange without inducing a rapid exchangeable component, the intracellular cholesterol transport protein SCP-2 dramatically facilitated DHE exchange by decreasing the half-time over two-fold and inducing the formation of a second DHE pool with very short, nearly 22-fold shorter, half-time, thereby nearly tripling the total fraction of sterol available for exchange under the experimental conditions.

Effect of SCP-2 on sterol exchange between crystalline dehydroergosterol and model membranes of large unilamellar vesicles

Large unilamellar vesicles consisting of 55 mol% POPC: 35 mol% Chol: 10 mol% PS were made by extrusion as described in the Methods. The exchange assay previously described was used to examine the ability of SCP-2 to enhance sterol exchange from out of a crystalline domain with that of model membrane associated cholesterol. DHE was added to 10mM PIPES buffer at pH 7.2 to form a 1.5 μ M solution of DHE. The aqueous dispersion of microcrystalline DHE (not shown) gave a baseline anisotropy of $r = 0.09$ and remain unchanged over a 4 h period similar to Fig. 4A. This experiment was repeated in the presence of 1.5 μ M SCP-2 (not shown), which also was unchanged over a 4 h time period. However, when the 55:35:10 (POPC:CHOL:PS) LUVs were added in 10-fold excess to the 1.5 μ M DHE microcrystalline dispersion, the anisotropy began to slowly rise with time indicative of sterol exchange as observed in Fig. 5A (closed circles). After 4 h, a spectrum of the solution was acquired which revealed a significant drop in the amount of crystalline DHE (Fig. 5B) as revealed by the ratio of 455 nm/375 nm = 0.82. As shown in Table 3 a single half-time component of 813 ± 1 min was calculated with the fraction fixed to 1 at infinitely long time. This was justified by the near linear slope of the curve over the 4 h time period. The initial rate calculated was very close to that of the BSA mediated and spontaneous DHE to cholesterol crystal exchange. Another solution of 1.5 μ M DHE microcrystalline dispersion was made in PIPES buffer, after 10 minutes the acceptors (10 $\times$ LUVs) was added along with SCP-2 for a final protein concentration of 1.5 μ M. This created a significant increase in anisotropy over the period of the 4 h as seen in Fig. 5A (open circles) with a corresponding increase in monomeric DHE content as seen in the emission spectrum (Fig. 5C) acquired immediately after the exchange time period. This spectrum showed very little excimeric emission as demonstrated by the low ratio of 455 nm/375 nm = 0.18. As shown in Table 3, a long half-time component of 133 ± 1 and an extremely short half-time of 0.31 ± 0.07 were calculated. The respective fractions of 0.8684 ± 0.0007 and 0.0314 ± 0.0002 revealed that the majority of the exchangeable sterol was in the slower fraction but 3% was located in a rapid exchangeable fraction which contributed to an initial rate of 40 ± 10 pmol/min. The addition of SCP-2 to the sterol exchange involving DHE microcrystals and LUVs boosted the initial rate 92-fold. The overall exchangeable sterol was 90% of the total sterol, which was clearly evident of the spectrum acquired after 4 h (Fig. 5C).

Effect of SCP-2 on nonfluorescent mechanisms in mixtures of crystalline cholesterol and model membranes of large unilamellar vesicles

In order to determine if the time-dependent enhancement of anisotropy was independent of the fluorescent sterol DHE and potentially a result of changes in residual scatter, several control experiments were performed using the same experimental and instrumental parameters but

with cholesterol crystal donors. First, the anisotropy of cholesterol crystals in PIPES buffer was monitored for 4 h. Second, the anisotropy of the mixture of cholesterol crystal donors in the presence of 10 fold excess of LUV cholesterol acceptors was monitored for 4 h. Last, the anisotropy of the mixture of cholesterol crystal donors in the presence of 10 fold excess of LUV acceptors and SCP-2 was monitored for 4 h as shown in Fig. 6. In all three cases, the anisotropy of the weak residual light scatter leaking through the KV389 filter remained constant over time implying that the changes in anisotropy in the case of DHE were not a scatter artifact involving crystals, LUVs, and the protein.

Effect of SCP-2 on sterol exchange between crystalline dehydroergosterol and biological membranes of three types: plasma membranes as well as the sub-fractions: CAVCR and non-CAVCP

Biological membranes in the form of vesicles were isolated by previously established methods and used to determine the significance of crystalline sterol domain exchange in complex systems. The plasma membranes of MDCK cells were isolated using differential (Percoll) centrifugation with subsequent sub-fractionation into two portions, CAVCR and non-CAVCP, through the use of concanavalin A affinity purification technique as described in Methods and characterized in studies involving diverse cell types (42,71-73,90). The cholesterol-rich sub-fraction (CAVCR) also contained proteins such as caveolin-1 and GM1 gangliosides as opposed to the cholesterol-poor sub-fraction (non-CAVCP). They were also highly purified and relatively free of contamination from endoplasmic reticulum.

Baseline anisotropy of DHE microcrystals in buffer alone was again essentially unchanged over a 4 h time period. The anisotropy of the 1.5 μ M DHE microcrystals in the presence of 10-fold excess of MDCK PM acceptor membrane vesicles showed no change over 4 h (Fig. 7A) and the spectrum acquired after the 4 h showed almost no change in the spectrum (not shown). However, with the same conditions and including 1.5 μ M SCP-2 at the time of addition of acceptors changed the dynamics completely (Fig. 7A). The anisotropy increased rapidly in nearly 4 h, corresponding to a single large exchangeable fraction of 0.9431 ± 0.0006 with a half-time of 72.9 ± 0.6 min (Table 3). The emission spectrum at 4 h (Fig. 7D) revealed mostly monomeric type emission as evidenced by the emission ratio of $455 \text{ nm}/375 \text{ nm} = 0.2$. The CAVCR sub-fraction of the MDCK PM also exhibited enhanced exchange in the presence of 1.5 μ M SCP-2 as shown in Fig. 7B (closed circles) albeit with the introduction of two components, one slow and one fast. The slow component resulted in a half-time of 270 ± 20 min with an exchangeable fraction of 0.299 ± 0.003 and the fast component in a half-time of 3.7 ± 0.3 min albeit a small fraction of 0.0453 ± 0.0002 . No changes were evident in the spontaneous exchange of the CAVCR sub-fraction without SCP-2 as shown in Fig. 7B (open circles). As previously observed in the PM, the spectrum after the exchange in the presence of SCP-2 was that of mostly monomeric DHE with an emission ratio of $455 \text{ nm}/375 \text{ nm} = 0.3$ (Fig. 7E). Sterol exchange, whether spontaneous or with SCP-2, was nonexistent in the non-CAVCP sub-fraction of the PM (Fig. 7C) with the spectrum (Fig. 7F) revealing a crystalline type emission with an emission ratio of $455 \text{ nm}/375 \text{ nm} = 1.5$. Herein SCP-2 provided greater enhancement of sterol transfer within the first hour as compared to an earlier study using CAVCR membrane vesicles containing DHE as donors to similarly obtained cholesterol containing acceptors (71). Also, SCP-2 did not significantly enhance sterol exchange between DHE containing donor non-CAVCP acceptor vesicles (71).

Multi-photon laser scanning microscopy of dehydroergosterol monohydrate

DHE in a solution of 90% ethanol and 10% water was evaporated at room temperature on chambered cover glass and imaged by multi-photon laser scanning microscopy using the instrumental setup described in the Methods. Many microcrystals of DHE monohydrate were formed as seen by the small fluorescent plate structures (Fig 8). Changes in shape and sizes

occurred depending upon conditions such as concentration, temperature, and water content and subsequently rate of evaporation. DHE in anhydrous ethanol evaporated under nitrogen, typically formed longer structures as observed in cholesterol (20).

Multi-photon imaging of crystalline dehydroergosterol disappearance in SCP-2 overexpressing cells as compared to control L-cells

L-cells and L-cells overexpressing SCP-2 were cultured as described in the Methods. After supplementing the cells with crystalline DHE for 24 h, cells were washed and serum containing media was added. Both control and SCP-2 overexpressing cells with phagocytosed crystals of DHE were immediately imaged by multi-photon microscopy using dual channel detection as described in the Methods at time $t = 0$. Power, gain, and black level were adjusted initially to minimize photobleaching, saturation, and dynamic range and maintained throughout the time based experiment. After the instrumental acquisition parameters were set appropriately, images of the autofluorescence of L-cells were acquired. Due to the brightness of the emission of microcrystalline DHE, no autofluorescence was observable in either the blue channel (Fig. 9A) or UV channel (Fig. 9B). Similar images (not shown) of the SCP-2 overexpressing cells were acquired. Images of the DHE emission from microcrystals of DHE in control cells and cells overexpressing SCP-2 were obtained by collecting the fluorescence in the blue and UV channels at $t = 0$. Fig 9C,D were multiphoton images acquired of the microcrystalline DHE in L-cells overexpressing SCP-2 at the initial time point. Subsequently, cells on several chambered cover glass were imaged at 12 h increments using multi-photon laser scanning microscopy using both channels. Representative images acquired from the blue (Fig. 9E) and UV channels (Fig. 9F) reveal the character of the remaining DHE in L-cells overexpressing SCP-2 after 60 hrs. Integrated intensity measurements were made of the dual channel acquisition images of cells across multiple regions of each slide and the mean integrated intensity was plotted as a function of time for both SCP-2 overexpressing and control cells (Fig. 10A). The rate of disappearance of DHE crystalline fluorescence (Fig. 10A) appeared linear over the period of 60 h for both cell types and did not appear significantly different between cell types due to large variability especially in the control L-cells. The SCP-2 overexpressing cells took up more microcrystals and trended toward faster disappearance as compared to the control cells. The emission at in the UV channel (375 nm) also decreased more rapidly even than that of the blue channel (455 nm) as seen in the graph of the ratio of the blue channel to UV channel (455 nm emission/375 nm emission) over time (Fig. 10B); which implied that at longer time points, monomeric DHE was distributed faster throughout the cellular membranes with a substantial portion effluxed out of the cell (89).

DISCUSSION

Excess cholesterol is cytotoxic and can result in deleterious accumulation of crystalline cholesterol within the cell (10-12). While increased excretion, intracellular esterification of cholesterol, and reduced hydrolysis of cholesteryl esters can in part prevent toxicity due to excess cholesterol (rev. in (13), other factors may exist to enhance dissolution of crystalline cholesterol and transport from the cell. Early studies with macrophages, NPC fibroblasts, and hypercholesterolemic rats suggested a potential role for intracellular sterol binding/carrier proteins such as sterol carrier protein-2 (SCP-2) (15,30-32). In fact SCP-2 has been shown to enhance transfer of DHE from out of microcrystals and exchange with cholesterol within intact lysosomal membranes (38). This was somewhat surprising since even though DHE microcrystals appeared in large quantities of lysosomes as determined by fluorescence imaging/colocalization and isolation of intact lysosomes, SCP-2 has been observed in only very small amounts within lysosomes. Since cells can accumulate excess cholesterol in microcrystalline patches within the plasma membrane (10), a part of the current study was undertaken with dehydroergosterol, a naturally-occurring fluorescent sterol analogue, and

purified SCP-2 to determine if this protein enhanced the transfer/exchange of sterol between crystals and between crystals and membrane acceptors, especially plasma membranes and associated membrane domains. Together the data presented herein provide new insights on the potential role of SCP-2 in dissolution of crystalline sterol and transfer of crystal derived sterol to cellular derived membrane fractions of enriched cholesterol and caveolin-1.

At the outset, the crystal structure of dehydroergosterol monohydrate was determined for the first time from DHE synthesized with a purity >99%. Comparison with the crystal structure of cholesterol monohydrate reveals overall similarities such as its organization into a bilayer system albeit with subtle differences, suggesting that it would be a suitable donor for SCP-2-mediated sterol transfer. Similar to ergosterol monohydrate, dehydroergosterol monohydrate crystallizes in the space group $P2_1$ with two molecules in the asymmetric unit while cholesterol monohydrate crystallizes in the space group $P1$ with four molecules in the asymmetric unit. If the unit cell of dehydroergosterol monohydrate was doubled and the space group reduced to $P1$ then the transformed unit cell of dehydroergosterol monohydrate would be comparable to cholesterol monohydrate. Although, cholesterol monohydrate has triclinic symmetry, it does have some pseudo-symmetry such that the cholesterol monohydrate can easily be mistaken for monoclinic (81,82). The low temperature monohydrate crystalline structure of ergosterol was quite similar to the reduced form (Table 1) where the main effect of the addition of the two double bonds (Fig. 1) forming the conjugated system brought about the shortening of the longest axis c and with the decrease in the angle β bringing it closer to approximating an orthorhombic system. The space group reduced cell parameters of dehydroergosterol monohydrate measured to be slightly smaller ($a = 9.9747(11)$ Å, $b = 7.4731(9)$ Å, $c = 34.054(4)$ Å, $\alpha = 90^\circ$, $\beta = 92.970^\circ(2)$, $\gamma = 90^\circ$) than those of cholesterol monohydrate ($a = 12.39$ Å, $b = 12.41$ Å, $c = 34.36$ Å, $\alpha = 91.9^\circ$, $\beta = 98.1^\circ$, $\gamma = 100.8^\circ$) as reported previously (81). In comparing dehydroergosterol monohydrate to the cholesterol monohydrate reported by Craven (81,82) the intermolecular distance between the centroids of the staggered ring system for dehydroergosterol monohydrate [$5.50(1)$ Å] was significantly shorter than cholesterol monohydrate [$6.33(1)$ Å]. Consistent with previous measurements of force-area isotherms of monolayers formed using mixtures of either high mol % dehydroergosterol or cholesterol with POPC, the density of dehydroergosterol monohydrate ($\rho_{\text{DHE}} = 1.081$ mg/m³) was slightly larger, with a ratio of $\rho_{\text{DHE}}/\rho_{\text{Chol}} = 1.034$, than that of cholesterol monohydrate ($\rho_{\text{Chol}} = 1.045$ mg/m³) (38,81). Taken together, these data would suggest that dehydroergosterol was arranged in a slightly closer packed configuration than cholesterol.

DHE in its crystalline form yields enhanced red shifted emission reminiscent of the excimeric emission of pyrene although with much less increase in fluorescence and some spectral structure. Pyrene forms crystals that are monoclinic with four molecules in a unit cell and belong to the space group $P2_1/a$ such that the pyrene molecules are arranged as sandwich dimers, forming the basis of the excimer. Such a parallel arrangement observed of the stacking of parallel dehydroergosterol molecules could also form the basis of excimeric sandwich dimers although at interplanar distances ~ 1.5 fold greater than in pyrene crystals. The larger distance in the DHE crystal could explain the lower amount of enhanced emission from monomer to excimer as compared to the pyrene crystal. Also, the vibrational level structure observed in the difference spectra (crystalline — monomer) could indicate a somewhat longer-lived ground state dissociative dimer. At the present time, the enhanced red-shifted emission seems to be relegated to the crystalline form as this spectral characteristic has not been observed in highly concentrated solutions (not saturated and with no observable crystals) of DHE in ethanol. As such the existence of such enhancement in emission has proven to be a very useful tool to detect the existence of crystalline form in biological systems (91,92).

Kinetic experiments, utilizing anisotropy measurements of aqueous dispersions of DHE microcrystals in the vicinity of 10-fold excess of acceptors, suggested that the addition of

SCP-2, but not albumin, significantly enhanced disruption of the DHE microcrystalline structure when in the presence of excess cholesterol. Of greater significance, in experiments with model membranes containing POPC, cholesterol, and DOPS, SCP-2 enhanced exchange of sterol between microcrystals and LUVs nearly 92 fold more in the initial rate than the spontaneous exchange, even affecting two dynamic pools of cholesterol within the model membrane acceptors. The question remains, however, whether model membrane system studies would reflect the DHE transfer between microcrystals and biological membranes such as the plasma membrane.

The plasma membranes of many types of cells contain specialized cholesterol-rich microdomains (rev. in (13,18)). The localization of the protein caveolin to these regions can result in membrane invaginations (50-100 nm) which are morphological features visualized by electron microscopy and defined as caveolae. By selectively partitioning specific lipids and proteins, these membrane regions represent a nexus organizing multiple cell membrane functions such as reverse cholesterol transport, cell recognition, signaling, immune function, and potocytosis (rev. in (4,9,93-97)). Although plasma membrane cholesterol-rich microdomains such as caveolae are important not only in normal cell physiology, aberrant microdomains are associated with metabolic diseases (cardiovascular, diabetes, obesity, Niemann-Pick C disease, Alzheimer's disease, as well as others) (19-26), pathogen entry, and the action of many bacterial and viral toxins (98-104). In order to better understand the potential role of such microdomains in the removal of crystalline sterol, kinetic experiments were repeated with vesicles derived from the plasma membrane of MDCK cells: (i). plasma membrane acceptors and (ii). affinity-purified acceptors consisting of CAVCR and non-CAVCP membrane vesicles sub-fractionated from plasma membrane isolation. Although the CAVCR sub-fraction was previously shown to contain proteins such as caveolin-1 and GM1 that are typically considered caveolae proteins and the non-CAVCP were shown to be void of these proteins, the argument remains at the edge of the definitive due to the invasive nature of the isolation (71,73,105). Whether or not the cholesterol-rich vesicles can truly be considered to behave similarly to intact caveolar membranes, the two sub-fractions respectively represent biological derived membrane substrates with and without a complement of active proteins thought to be involved in sterol uptake/efflux (rev. in (9,106)). As such, these fractions were used to characterize the interaction of crystalline sterol and active proteins in a biologically relevant lipid microsystem. In the plasma membrane fraction, a rather large amount (94%) of the sterol was exchangeable with a half-time on the order of 73 min, a value somewhat faster than that of MDCK membrane fractions but typical of other organellar types (71,107). Due to the large size of this rapid exchangeable pool and variability in isolations, it is possible that a much smaller fast component was obscured. Noticeably, in the binding sub-fraction (CAVCR) of the MDCK plasma membrane, a significantly faster component emerged that represented about 5% of the total sterol and 13% of the total exchangeable pool. However, only about 35% of the total sterol was exchangeable compared to approximately 58% when using plasma membrane binding fraction donors as well (71). Similar to the plasma membrane vesicles, in LUVs almost all of the sterol was available for exchange in both the spontaneous and the SCP-2 enhanced. Two dynamic pools were observed wherein the majority (87%) pool had exchange rates on the order of a typical cholesterol-rich microdomain sub-fraction and a small pool (3%) had a very fast exchange rate (half-time on the order of seconds). Significant enhancement, as a result of the addition of SCP-2, occurred for the transfer of DHE out of microcrystals into membranes vesicles, both model and cellular except for the non-CAVCP, as might be expected at the outset. Although the largest bulk transfer occurred from plasma membrane vesicles, the CAVCR showed significant decrease in half-time but with less exchangeable sterol availability, possibly due to an intrinsic protein-cholesterol binding interaction (e.g. caveolin).

Multi-photon laser scanning microscopy with its optical sectioning capability has proven indispensable in observing naturally occurring fluorophores like DHE that absorb in ultraviolet

wavelengths. With this technique, microcrystals of DHE have been observed to incorporate into L-cells (38). This was not surprising since L-cells have been shown to take up latex beads nearly 2 μm in diameter (108). Noninvasive spectroscopic imaging measurements revealed that living cells work to reduce the amount of intracellular crystalline sterol and showed that lysosomes play a major role in this process but little was known about whether SCP-2 might aid in the intracellular dissolution process (38). L-cells overexpressing SCP-2 were used to determine the rate of disappearance of microcrystalline DHE monohydrate in real-time as compared to control cells. Cellular monomeric DHE that remained within the cell localized in lipid storage droplets and intracellular membranes with substantial accumulation in the plasma membrane as formerly observed in L-cells (38,58).

Previous studies have demonstrated the ability of protein interaction in facilitating uptake of aqueous dispersions of cholesterol aggregates. In cells overexpressing the scavenger receptor class B type I (SR-BI) it was shown that SR-BI effected uptake of free radiolabeled cholesterol ($[^3\text{H}]$ cholesterol) to stimulate cholesterol esterification after a 5 h incubation period with similar effectiveness as lipoproteins (109). Moreover, SR-BI was shown to facilitate uptake of cholesterol from mixed bile salt micelles (110).

In conclusion, not only the low solubility of cholesterol in aqueous media but also the build up of crystalline patches in cellular membranes presents many challenges to biological systems. However, small lipid binding proteins such as SCP-2 in combination with specialized regions of lipid-protein systems can facilitate and mediate the exchange of sterol though sequestered in microcrystalline form in order to adequately transport and access the sterol for further utilization. Therefore the observed enhanced transfer effects with SCP-2 reveal the possibility of lipid-protein processes to remediate cholesterol aggregation within the cell. This was demonstrated herein through the use of the naturally occurring fluorescent cholesterol analog, DHE, in monohydrate crystalline structures somewhat resembling that of cholesterol monohydrate but with unique fluorescent spectral properties.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

DHE, dehydroergosterol; BHT, butylated hydroxytoluene; APCI, (atmospheric pressure chemical ionization) mass spectroscopy; HPLC, high performance liquid chromatography; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; DOPS, 1,2-dioleoyl-*sn*-glycero-3-[phospho-L-serine]; CAVCR, caveolin-1 and cholesterol-rich affinity purified sub-fraction; non-CAVCP, cholesterol-poor affinity purified sub-fraction without caveolin-1; MPLSM, multi-photon laser scanning imaging microscopy.

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Figure 1. The structural differences between ergosterol and fluorescent reduced ergosterol (DHE)
A. Ergosterol (ergosta-5,7,22-trien-3 β -ol) contains three double bonds. **B.** Reduced ergosterol (ergosta-5,7,9(11),22-tetraen-3 β -ol) contains an additional double bond located between carbon 9 and carbon 11 of the third ring, forming a conjugated triene system.

Figure 2. Crystal structure of dehydroergosterol monohydrate

The unit cell consists of 4 dehydroergosterol monohydrate molecules, two (A and B) of which are unrelated by crystal symmetry. **A.** 3-D ball and stick representation of the orientation of the A and B molecules in the crystallographic unit cell. The hydrogen atoms on the water molecules were disordered and are not shown. **B.** 3-D space-filling representation of the orientation of the A and B molecules in the crystallographic unit cell. Molecules of oxygen are shown in dark gray, hydrogen in white, and carbon in light gray.

Figure 3. Excitation and emission anisotropy of DHE

A. Plots of the anisotropy versus excitation wavelength over the region 280-350 nm for emission collected at 375 nm (open circles) and 425 nm (closed circles). **B.** Plot of emission anisotropy over the region 370-550 nm, resulting from excitation at 324 nm. Bandwidths were 5 nm for both excitation and emission monochromators.

Figure 4. Anisotropy and spectral measurements of microcrystal disruption involving DHE and cholesterol

Exchange of sterol was monitored using polarized light as described in the Methods with excitation at 324 nm. **A.** 2.5 μM DHE was added to buffered 10mM PIPES (pH. 7.2) to form microcrystals. The polarization was monitored for 225 minutes and converted to anisotropy. This curve represents the donor only curve and remains flat over the time period. **B.** Emission spectrum of the DHE microcrystals after baseline anisotropy measurement of (**A**), approximately 4 hrs. **C.** Time-dependent changes in anisotropy involving donor 2.5 μM DHE as a result of the addition of 25 μM cholesterol to the solution as a 10 fold excess of acceptor. **D.** Emission spectrum after measurement of the spontaneous sterol exchange in (**C**), at

approximately 4hrs. **E.** Sterol exchange between a donor 2.5 μM DHE microcrystal dispersion in the presence of 25 μM cholesterol microcrystal dispersion with the addition of 2.5 μM BSA. **F.** Emission spectrum acquired after the sterol exchange in (**E**) at approximately 4hrs. **G.** Sterol exchange between a 2.5 μM DHE microcrystal dispersion (donors) in the presence of 25 μM cholesterol microcrystal dispersion (acceptors) with the addition of 2.5 μM SCP-2. **H.** Emission spectrum of the combination in (**G**) acquired at 4 hrs.

Figure 5. Sterol exchange between donor DHE microcrystals and large unilamellar vesicles (LUVs) Large unilamellar vesicles (55 mol % POPC: 35 mol % cholesterol: 10 mol % PS) were prepared as described in the Methods. **A.** Spontaneous and SCP-2 mediated exchange of sterol between a 1.5 μ M solution of DHE microcrystals (donor) and a 10-fold excess of LUVs (acceptors) in PIPES buffer were monitored by anisotropy measurements with excitation at 324 nm. **B.** Spectrum acquired after 4 h of the spontaneous exchange (DHE + 10-fold excess LUV). **C.** Spectrum acquired after 4 h of the SCP-2 mediated exchange (DHE + 10-fold excess LUV + 1.5 μ M SCP-2).

Figure 6. Time dependent anisotropy measurements of cholesterol microcrystals with large unilamellar vesicles (LUVs)

Large unilamellar vesicles (55 mol % POPC: 35 mol % cholesterol: 10 mol % PS) were prepared as described in the Methods. **A.** Using the same instrumental setup for measuring the anisotropy of DHE (Figure 4), residual scatter was monitored for approximately 4 h using a 1.5 μ M solution of cholesterol microcrystals (donor) and a 10-fold excess of LUVs (acceptors) in PIPES buffer.

Figure 7. Sterol exchange between donor DHE microcrystals and acceptors of biological plasma membrane fractions and sub-fractions

Plasma membranes were isolated from Madin-Darby canine kidney (MDCK) cells followed by sub-fractionation of the plasma membrane isolation, to yield CAVCR and non-CAVCP vesicles as described in the Methods. Sterol exchanges were performed in a quartz cuvette with a 1.5 μ M solution of DHE microcrystals as donors and a 10-fold excess of acceptor membrane vesicles in 10 mM PIPES buffer at pH 7.2. The anisotropy was measured with an ISS PC1 spectrofluorometer using excitation at 324 nm and the kinetic curves plotted for the spontaneous and SCP-2 mediated exchange of sterol from the donor DHE microcrystals to the following acceptors: **A.** plasma membrane; **B.** CAVCR; and **C.** non-CAVCP. After a 4 h sterol exchange assay, spectra were acquired at 324 nm excitation to determine the structural form of the DHE. Spectra were plotted for the SCP-2 mediated exchanges (DHE donor microcrystals + 10-fold excess acceptors + 1.5 μ M SCP-2) for the following acceptors: **D.** plasma membrane

after 4 h showing mostly monomeric DHE; **E.** CAVCR after 4 h showing mostly monomeric DHE; **F.** non-CAVCP after 4 h showing microcrystalline DHE.

Figure 8. Multi-photon laser scanning microscopy of DHE monohydrate microcrystals

The microcrystals were formed by evaporation from a solution of DHE in 90% ethanol and 10% water onto a chambered cover glass. The microcrystals were then imaged through a Zeiss 63X plan-Apochromat 1.4 NA oil immersion objective using a Bio-Rad MRC-1024 MP laser scanning microscopy imaging system with a Coherent titanium:sapphire femto-second mode-locked laser tuned to an excitation wavelength of 900 nm. This grayscale image of the plate-like structures represents the DHE crystalline enhanced fluorescence filtered through a Chroma Technology 455/30 nm dichroic filter. White scale bar in the lower right-hand corner of the image represents 4 μm .

Figure 9. The effect of SCP-2 overexpression on DHE microcrystals in living cells

Living cells on chambered cover glass were incubated with DHE microcrystals for 24 h. Cells were washed with PBS three times and the replaced with serum containing media. The cells were immediately imaged at $t = 0$ using multi-photon laser scanning microscopy imaging. Multi-photon excitation of the DHE was accomplished at 900 nm with simultaneously mostly crystalline DHE fluorescence emission was collected using a D455/30 nm filter (blue channel) and mostly monomeric DHE fluorescence emission was collected using a D375/50 nm filter (UV channel). The cells on chambered cover glass were returned to the incubator and subsequently imaged every 12 h up to 60 h. **A-B.** Image of L-cells without DHE labeling (autofluorescence) collected using the blue channel (**A**) and the UV channel (**B**). The gain and

black levels were adjusted to reduce the autofluorescence to near zero as seen in the images (**A-B**). Fluorescence emission of DHE microcrystals in living cells overexpressing SCP-2 as imaged in the blue channel (**C**) and UV channel (**D**) at time $t = 0$. Fluorescence emission of DHE microcrystals in living cells overexpressing SCP-2 as imaged in the blue channel (**E**) and UV channel (**F**) at time $t = 60$ h. Scale bar in lower right corner represents 10 μm .

Figure 10. Disappearance of microcrystalline DHE from L-cells and L-cells overexpressing SCP-2
A. The background corrected integrated intensities obtained from images of DHE emission collected through the D455/30 nm filter (blue channel) were plotted for both control L-cells and cells overexpressing SCP-2. **B.** The ratio of the background corrected integrated intensities of monomer to crystalline that were acquired using the D455/30 nm filter (blue channel) and the D375/50 nm filter (UV channel) were plotted for both control L-cells and L-cells overexpressing SCP-2. Each point was derived from mean integrated intensities obtained from collective measurements of ~50 cells and corrected for residual background autofluorescence; the error bars represent the standard error.

Table 1

Comparison of low temperature structure (173K) of Ergosterol¹ and the low temperature structure (110K) of Dehydroergosterol (reduced Ergosterol)².

Formula	C ₂₈ H ₄₄ O ₁ H ₂ O	C ₂₈ H ₄₂ O ₁ H ₂ O
Formula weight	414.6	412.6
Space Group	P2 ₁	P2 ₁
Cell Constants		
A	9.902(1)	9.975(1) Å
B	7.518(1)	7.4731(9) Å
C	34.657(7)	34.054(4) Å
β	94.25(3)	92.970(2)°
V	2572.9(5)	2535.1(5) Å ³
Z	4	4
D _{cal}	1.071	1.081
R _f [I>2σ(I)]	6.8	6.9

¹Lobkovsky, 2000 private communication

²This work

Table 2

Crystal data and structure refinement for dehydroergosterol

Empirical formula	C ₂₈ H ₄₄ O ₂
Formula weight	412.63
Temperature	110(2) K
Wavelength	0.71073 Å
Crystal system	Monoclinic
Space group	P2 ₁
Unit cell dimensions	a = 9.9747(11) Å α = 90°. b = 7.4731(9) Å β = 92.970(2)°. c = 34.054(4) Å γ = 90°.
Volume	2535.1(5) Å ³
Z	4
Density (calculated)	1.081 Mg/m ³
Absorption coefficient	0.065 mm ⁻¹
F(000)	912
Crystal size	0.20 × 0.20 × 0.10 mm ³
Theta range for data collection	2.10 to 24.99°
Index ranges	-11 ≤ h ≤ 11, -8 ≤ k ≤ 8, -40 ≤ l ≤ 40
Reflections collected	32269
Independent reflections	8865 [R(int) = 0.0563]
Completeness to theta = 24.99°	99.4 %
Absorption correction	None
Max. and min. transmission	0.9935 and 0.9870
Refinement method	Full-matrix least-squares on F ²
Data / restraints / parameters	8865 / 1 / 542
Goodness-of-fit on F ²	1.041
Final R indices [I > 2σ(I)]	R1 = 0.0688, wR2 = 0.1766
R indices (all data)	R1 = 0.1180, wR2 = 0.2142
Absolute structure parameter	0(3)
Largest diff. peak and hole	0.505 and -0.331 e.Å ⁻³

Initial rates and kinetic multi-exponential analysis of molecular sterol exchange

Fluorescence polarization exchange curves for DHE sterol transfer from DHE donor to cholesterol crystals and membrane vesicles. The exchanges involving LUV, PM, CAVCR, and non-CAVCP were measured in the absence or presence of SCP-2 (1.5 μ M) followed by determination of initial rates and kinetic analysis as described in Methods. Half times $t^{1/2}_1$ and $t^{1/2}_2$ were in minutes with the corresponding fractions, f_1 and f_2 , representing the fractions due to the exchangeable components. Values represent the mean $\pm$ SE (n=3-4).

Donor-Acceptor	Protein Added	Initial Rate (nmol/min)	$t^{1/2}_1$ (min)	f_1	$t^{1/2}_2$ (min)	f_2
DHE-Chol	None	0.3 $\pm$ 0.06	130 $\pm$ 20	0.057 $\pm$ 0.001	-	-
	BSA	0.45 $\pm$ 0.03	60 $\pm$ 2	0.0400 $\pm$ 0.0002	-	-
	SCP-2	8.8 $\pm$ 0.7	74 $\pm$ 4	0.0867 $\pm$ 0.0003	6.1 $\pm$ 0.3	0.0732 $\pm$ 0.0006
DHE-LUV	None	0.435 $\pm$ 0.002	813 $\pm$ 1	1 ^a	-	-
	SCP-2	40 $\pm$ 10	133 $\pm$ 1	0.8684 $\pm$ 0.0007	0.31 $\pm$ 0.07	0.0314 $\pm$ 0.0002
DHE-PM	None	-	-	-	-	-
	SCP-2	4.67 $\pm$ 0.06	72.9 $\pm$ 0.6	0.9431 $\pm$ 0.0006	-	-
DHE-CAVCR	None	-	-	-	-	-
	SCP-2	3.9 $\pm$ 0.1	270 $\pm$ 20	0.299 $\pm$ 0.003	3.7 $\pm$ 0.3	0.0453 $\pm$ 0.0002
DHE-non-CAVCP	None	-	-	-	-	-
	SCP-2	-	-	-	-	-

^a Value was fixed during fit.