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LIPID TRANSFER PROTEIN BINDING OF UNMODIFIED NATURAL LIPIDS AS ASSESSED BY SURFACE PLASMON RESONANCE METHODOLOGY

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Abstract

A new approach for analyzing lipid-lipid transfer protein interactions is described. The transfer protein is genetically engineered for expression with a C-terminal biotinylated peptide extension (AviTag®). This allows protein anchoring to a streptavidin-coated chip for surface plasmon resonance (SPR)-based assessment of lipid binding. Sterol carrier protein-2 (SCP-2), involved in the intracellular trafficking of cholesterol, fatty acids and other lipids, was selected as the prototype. Biotinylated SCP-2 (bSCP-2) was expressed in *E. coli*, purified to homogeneity by mutated streptavidin (SoftLink®) affinity chromatography, and confirmed by mass spectrometry to contain one biotin group at the expected position. Intermembrane [¹⁴C]cholesterol transfer was strongly enhanced by bSCP-2, demonstrating that it was functional. Using bSCP-2 immobilized on a Biacore streptavidin chip, we determined on- and off-rate constants along with equilibrium dissociation constants for the following analytes: oleic acid, linoleic acid, cholesterol, and fluorophore (NBD)-derivatized cholesterol. The dissociation constant for NBD-cholesterol was similar to that determined by fluorescence titration for SCP-2 in solution, thus validating the SPR approach. This method can be readily adapted to other transfer proteins and has several advantages over existing techniques for measuring lipid binding, including (i) ability to study lipids in their natural states, i.e. without relatively large reporter groups; and (ii) ability to measure on- and off- rate constants as well as equilibrium constants.

Keywords

SPR; sterol carrier protein-2; cholesterol; fatty acids; association and dissociation rate; equilibrium constant

INTRODUCTION

Intracellular trafficking of fatty acids and other lipids is facilitated by various transfer proteins, which play an important role in lipid metabolism and in membrane biosynthesis and refashioning [1–4]. Lipid transfer proteins are also crucial in plasma, where they mediate phospholipid and cholesteryl ester translocation in connection with lipoprotein differentiation and maturation [5,6]. Among the three basic types of transfer protein identified in mammalian cells [3], 13.2 kDa sterol carrier protein-2 (SCP-2) has been studied most extensively since its

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isolation about 25 years ago [2]. SCP-2 is encoded by the SCP-x/pro-SCP-2 composite gene, expression of which gives 58 kDa SCP-x (~45 kDa representing peroxisomal 3-ketoacyl-CoA thiolase) and 15.2 kDa pro-SCP-2 [7,8]. Mature SCP-2 can be generated by post-translational C-terminal proteolysis of either pro-SCP-2 or SCP-x [7,8]. Also known as non-specific lipid transfer protein because it recognizes phospholipids, fatty acids, and fatty acyl CoAs in addition to cholesterol, SCP-2 is found in several subcellular compartments, most prominently in peroxisomes, but also in mitochondria, lysosomes, and cytosol [7,9]. Multiple site location is consistent with the idea that SCP-2 is involved in lipid trafficking between compartments for key metabolic requirements, e.g. in adrenocortical cells, facilitating cholesterol transfer from lipid droplet stores to mitochondria for steroid hormone biosynthesis [10].

Numerous studies with a variety of model systems ranging from unilamellar liposomes to isolated cellular organelles have shown that translocation of cholesterol and phospholipids from donor to acceptor membranes can be greatly accelerated by SCP-2 [11–13]. More recently, it was shown that SCP-2 can also enhance intermembrane transfer of peroxidized cholesterol and phospholipids, thereby expanding the damaging prooxidant range of these species [14,15]. With regard to mechanism of transfer enhancement, it appears that SCP-2 actually binds and transports donor membrane lipids rather than facilitates their departure without physically interacting with them [7]. The most convincing evidence for direct binding was obtained from fluorescence titration measurements in aqueous solution, using either natural or recombinant SCP-2 and fluorophore-tagged lipids like NBD-phosphatidylcholine or NBD-cholesterol [16,17]. Equilibrium dissociation constants (K_d) in the low nM to low μ M range have been reported for NBD or pyrene derivatives of cholesterol, phosphatidylcholine, and stearic acid [7]. The fluorophore groups of such conjugates facilitate binding measurements, but at the same time add considerable size to the lipids of interest, the molecular mass of NBD-stearate, for example, being nearly 40% greater than that of stearate. This factor, along with where the marker group is attached to a lipid, could significantly influence the latter's binding properties. In addressing this issue, we have developed a novel strategy for examining lipid-transfer protein interactions which eliminates the need for indicator groups. In this approach, a transfer protein is expressed with a selectively placed biotinylated peptide appendage, allowing it to be uniformly attached to a streptavidin chip for surface plasmon resonance (SPR)-based measurement [18–20] of lipid binding. Among the advantages of this approach is its very high sensitivity, which allows unsaturated fatty acids and certain other lipids to be studied below their critical micellar concentrations (CMC). In this report, we demonstrate the feasibility of this approach for determining lipid binding kinetics and equilibrium constants, using site-specifically biotinylated SCP-2 (bSCP-2) and selected fatty acids and sterols as the test system.

MATERIALS AND METHODS

General materials

Sigma Chemical Co. (St. Louis, MO) supplied the oleic acid and linoleic acid (each at least 99% pure), non-radioactive cholesterol, egg phosphatidylcholine (PC), dicetylphosphate (DCP), 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate hydrate (CHAPS), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), defatted bovine serum albumin (BSA), trypsin, desferrioxamine (DFO), EDTA, and Chelex-100 (50–100 mesh). The 100 kDa molecular mass cutoff (MWCO) filters were obtained from Millipore (Billerica, MA). Novagen (Madison, WI) supplied the *E. coli* strain BL21(DE3). NBD-cholesterol was from Molecular Probes (Carlsbad, CA). [4- 14 C]Cholesterol (51 mCi/ml), obtained from Amersham Life Sciences (Arlington Heights, IL), was HPLC-purified before use [21]. Avidity (Denver, CO) supplied the following: (a) pCAT7, a modified AviTag[®] construct consisting of a pET-28a plasmid with prolyl-rich semi-rigid hinge linker inserted before the C-terminal AviTag

segment, and (b) pBirA, a plasmid encoded for biotin ligase. Streptavidin SoftLink[®] resin was obtained from Promega (Madison, WI). Supplies for SPR analyses, including streptavidin (SA) sensor chips and P20 non-ionic detergent, were from Biacore Inc. (Piscataway, NJ).

Expression and isolation of recombinant SCP-2

E. coli bearing a plasmid encoded for 13.2 kDa human SCP-2 were grown in 1-liter of Luria-Bertani broth and at $A_{600} \sim 0.6$, protein expression was induced with 0.1 mM isopropyl β -D-thiogalactoside (IPTG) (20). Four hours later, cells were pelleted ($7500 \times g$, 10 min), resuspended in lysis buffer [0.1 M KH_2PO_4 , 1 mM DTT, 0.5 mM EDTA (pH 7.4)] and lysed by sonication. After removal of cellular debris by centrifugation, 4.5 ml of the supernatant was applied to a 5 ml HiTrap[®] SP column (Supelco, Bellefonte, PA) linked to an Akta-Prime FPLC system (Amersham-Pharmacia, Piscataway, NJ). SCP-2 was eluted at 25 °C, using a linear gradient of 0.1 M KCl in lysis buffer. Protein-containing fractions monitored by A_{280} were pooled and dialyzed at 4 °C against lysis buffer. Protein concentration was determined by Bradford assay [22] and purity checked by SDS-PAGE analysis. The latter was carried out using a NuPAGE 4–12% acrylamide/bis-acrylamide minigel from Invitrogen (Carlsbad, CA). Protein samples were reduced with dithiothreitol before electrophoresing and protein bands were visualized by staining with SimplyBlue (Invitrogen). Other details were as described [23].

Fluorescence titration of SCP-2 with NBD-cholesterol

A Model QM-7SE spectrofluorimeter from Photon Technology International (London, Ontario, CAN) was used for fluorescence titrations. Excitation was set at 460 nm (5 nm slit) and emission was scanned over the 500–600 nm range (7 nm slit). Fluorescence changes are represented in terms of integrated peak areas. In a typical run, 400 nM SCP-2 in 3.0 ml of 10 mM HEPES/25 μM EDTA/25 μM DFO/0.001% (w/v) CHAPS (pH 7.4) was placed into a quartz fluorescence cuvette thermostatted at 25 °C. Negligible volume aliquots of NBD-cholesterol (2.0 μM in dimethylformamide) were introduced and incubated for 4 min with slow stirring before measurements were taken. Running buffer without SCP-2 was titrated under identical conditions as a reference. Reference-corrected fluorescence (ΔF) was plotted as a function of increasing free NBD-cholesterol. A reciprocal plot of the data was used to obtain F_{max} and K_d values.

Expression, isolation and characterization of bSCP-2

The human SCP-2 gene from a plasmid stock [24] was used as a template for PCR amplification of SCP-2 cDNA. Forward (5'-GCGCCCATGGCTTCTGCAAGTGATGG-3') and reverse (5'-CCGGAAGCTTCCGAGCTTAGCGTTGCCTGG-3') primers (Integrated DNA Technologies, Coralville, IA) containing *Nco*I and *Hind*III restriction sites, respectively, were used. The cDNA was digested with *Nco*I and *Hind*III and ligated into the pET-28a plasmid encoded for a modified AviTag [25] at the SCP-2 C-terminus (pCAT7-scp2). DNA sequencing (carried out at the Protein and Nucleic Acid Facility of this institution) confirmed that the construct had the intended sequence for expression of SCP-2 with appended modified AviTag, the sequence of the latter being as follows: SLSTPPTPPSPSTPPTGLNDIFEAQKIEWHE. BL21(DE3) bacteria transformed with the pCAT7-scp2 and pBirA (biotin ligase-encoded) plasmids were grown to $\text{OD}_{600} \sim 0.7$ in Luria-Bertani broth and induced with 0.1 mM IPTG. Biotin ligase was expected to enhance biotin conjugation to the single lysine located six residues from the C-terminus [25]. Four hours after induction, the cells were harvested by centrifugation, resuspended in lysis buffer [50 mM Tris-HCl, 5 mM DTT, 1 mM EDTA, 5 % (v/v) glycerol (pH 7.5)] and lysed by sonication. The supernatant recovered after centrifugation ($10000 \times g$, 20 min), was passed through a 100 kDa MWCO membrane to remove biotin ligase and its natural substrate, biotin carboxy carrier protein (BCCP), from the system. The filtrate

was then applied three times in succession to a 5 ml column of SoftLink resin at 4 °C and eluted at 0.5 ml/min. SoftLink beads contain a mutated form of streptavidin, the K_d of which for biotin is $\sim 10^{-7}$ M, i.e. 10^8 -fold greater than that of natural streptavidin [26]. bSCP-2 was eluted with 5 mM biotin, fractions being collected every two minutes at a flow rate of ~ 0.6 ml/min. Protein-containing fractions were pooled, dialyzed against lysis buffer, then analyzed for protein content [22] and SDS-PAGE-assessed homogeneity [23]. MALDI-TOF mass spectrometric analyses of purified bSCP-2 were carried out at the Protein and Nucleic Acid Facility of this institution, using a Voyager-DE PRO system (Applied Biosystems, Inc.) and LTQ XL linear ion trap system (Thermo Electron, Corp.).

Transfer assay

The transfer activities of recombinant SCP-2 and bSCP-2 were determined by measuring the kinetics of [14 C]cholesterol translocation from small unilamellar vesicles (50 nm SUVs) to red cell ghost membranes [14,27,28]. Departure from SUV donors is the rate limiting step in the transfer process [27]. Stock SUVs consisting of 1.0 mM egg PC, 0.8 mM [14 C]cholesterol (~ 1 μ Ci/ml), and 0.02 mM DCP in bulk suspension were prepared by lipid extrusion, as described [27,28]. Unsealed ghosts were prepared by hypotonic lysis of human erythrocytes [27]. Assay mixtures (2.0 ml starting volume) typically contained 0.6 mM total ghost lipid, 0.06 mM total SUV lipid, and 5–10 μ M SCP-2 or bSCP-2 in PBS/0.1 mM EDTA/0.1 mM DFO (pH 7.4). The chelators were included to minimize any metal ion-catalyzed lipid or protein oxidation [14, 27,28]. At various times during transfer incubation at 37 °C, a 0.1 ml sample was removed, mixed with ice-cold PBS/EDTA/DFO, and centrifuged ($16000 \times g$, 10 min), after which 0.1 ml of the SUV-containing supernatant was analyzed by scintillation counting. Transfer rates were determined from plots of [14 C]cholesterol departure kinetics.

Preparation of analyte solutions

Stock solutions of ~ 70 mM oleic acid or linoleic acid were prepared by dissolving ~ 20 mg of the fatty acid in 1.0 ml of ice-cold acetone. An appropriate volume of each stock was diluted to 5.0 ml with running buffer [10 mM HEPES/25 μ M EDTA/25 μ M DFO/0.001 % (w/v) CHAPS (pH 7.4), except where indicated], bath-sonicated for 1–2 min at 25 °C and vortexed for 5 min to promote homogeneous solubilization. Total oleic or linoleic acid in this stock solution was below its reported critical micellar concentration (CMC) [29,30]. The actual concentration of free fatty acid was determined using the fluorescent probe ADIFAB2 (see below). This concentration was typically lower than expected, most likely because of fatty acid adsorption to working surfaces. Serial dilutions of the stock solution were then made with running buffer and subjected to SPR analysis. All of the preparative and analytical steps were carried out immediately before SPR runs.

An aliquot of stock NBD-cholesterol in 2-propanol was diluted to 5.0 ml with running buffer to give ~ 2.0 μ M NBD-cholesterol. After bath sonication for 10 min and vortexing for 5 min, the concentration of the solution was checked spectrophotometrically at 484 nm, using an extinction coefficient of $22,000 \text{ M}^{-1}\text{cm}^{-1}$ [31]. Several dilutions with running buffer were then made and subjected immediately to SPR analysis.

A stock solution of ~ 2.0 mM (0.1 mCi/ml) HPLC-purified [$4\text{-}^{14}\text{C}$]cholesterol in chloroform was diluted 100-fold in ice-cold acetone. A 0.2 ml aliquot of this solution was diluted to 5.0 ml with running buffer, sparged with argon for ~ 10 min to evaporate the acetone, then bath-sonicated for 15 min and vortexed for 5 min. All of these steps were carried out at 25 °C. The concentration of solubilized free cholesterol in this preparation was determined by scintillation counting, using a specific radioactivity of 51 Ci/mol (provided by Amersham Life Sciences). Immediately thereafter, diluted samples in running buffer were analyzed by SPR

Determination of fatty acid concentrations

Prior to SPR examination of oleic acid or linoleic acid binding, the concentration of free fatty acid was determined using the ADIFAB2 Kit from FFA Sciences (San Diego, CA). This approach allows concentrations of fatty acids below critical micelle limits to be determined [32]. ADIFAB2 is an acrylodan-labeled fatty acid binding protein which is sensitive to fatty acid uptake by virtue of a 457 to 550 nm shift in its fluorescence emission maximum. Using 375 nm excitation, one determines the concentration of free and protein-bound fatty acid in solution by measuring the ratio of 550 nm emission intensity to 457 nm emission intensity [32]. Excitation and emission slit widths on the spectrofluorimeter used were set at 2 nm and 4 nm, respectively. The emission intensity ratio was first measured for free ADIFAB2 (0.5 μ M) in 1.5 ml of 20 mM HEPES/140 mM NaCl/5 mM KCl/1 mM Na₂HPO₄ (pH 7.4) at 25 °C. A 100 μ l aliquot of oleic or linoleic acid solution was then added to the cuvette and the change in intensity ratio recorded. Using the equations provided by FFA Sciences [33], we calculated the free fatty acid concentrations used in SPR runs.

Immobilization of bSCP-2 on a streptavidin sensor chip

Affinity-purified bSCP-2 (~1 ml) was dialyzed exhaustively against Chelex-treated 10 mM HEPES (pH 7.4) containing 25 μ M each of EDTA and DFO. The protein was immobilized to the surface of one flow cell of a Biacore SA chip by injecting 50 μ l of a 1.5–1.7 μ M protein solution across this cell at a rate of 10 μ l/min. This typically produced a response increment of 1500 resonance units (RU), which corresponds to ~1.5 ng of protein/mm². A reference cell was generated by injecting 10 μ l of a 10 μ M biotin solution across a free flow cell over a 1-min period. The bSCP-2-containing flow cell received an identical injection of biotin to saturate any binding sites not occupied by the protein.

Surface plasmon resonance analyses

A Biacore-3000[®] instrument (Biacore AB, Uppsala, Sweden) was used for SPR measurements, all of which were carried out at 25 °C using a constant flow rate of 50 μ l/min. Analyte back transfer during the desorption stage of a run [34] was found to be minimal at this flow rate. Sensorgrams were processed and analyzed using the BIAevaluation software package (version 4.0.1) and a 1:1 (mol/mol) Langmuir binding model for determining rate constants. The fitting model also contained a floating term to correct for slight changes in the bulk refractive index of analyte solutions. SPR data were double-referenced [35], i.e. corrected for analyte solution responses as well as pure buffer responses, the former reflecting bulk refractive index changes and the latter any background instrumental “noise”. The typical injection sequence for an SPR run was as follows: (a) fatty acid or sterol solution (association phase); (b) running buffer (dissociation phase); (c) 0.4% (w/v) CHAPS in running buffer (regeneration phase); (d) running buffer (wash-out phase to remove excess CHAPS from injection needle and tubing prior to the next run). For each step, 100 μ l of lipid or buffer solution was injected over a 2-min period. The association, dissociation, and regeneration phases were monitored by measuring response changes (RU) over time. All analyses were carried out at least in duplicate.

RESULTS

Molecular characteristics of bSCP-2

As shown in Fig. 1, FPLC-purified recombinant SCP-2 appeared as a single band on SDS-PAGE, its M_r being ~13 kDa, as expected [7,8,11]. Expressed bSCP-2 isolated by SoftLink affinity chromatography also migrated as a single band (Fig. 1) and its observed size (M_r ~17 kDa) is consistent with the presence of a biotinylated AviTag extension (~3.5 kDa). Purified bSCP-2 was further characterized using MALDI-TOF mass spectrometry. Two post-translational modifications were confirmed: (a) cleavage of the requisite N-terminal

methionine by *E. coli* methionyl aminopeptidase [36], and (b) biotin incorporation at the lysyl residue of the AviTag segment. Examination of intact bSCP-2 revealed a molecular ion peak with m/z value of 16764.5. This agrees closely with the calculated molecular mass of bSCP2, 16769.2 Da, which takes into account the indicated post-translational modifications. A tryptic digest of bSCP-2 was also subjected to MALDI-TOF analysis. The entire protein was accounted for in the observed peptide fragments. Included among these was a major molecular ion of m/z value 3674.05. This is close to the calculated mass (3676.13 Da) for a biotinylated AviTag extension proteolytically cleaved at lysine 122, the penultimate C-terminal residue of human SCP-2 [11,25]. Using Ellman's reagent for thiol determination [37], we also showed that a bSCP-2 preparation contained 0.85 ± 0.05 mol cysteine/mol protein, which agrees with evidence [14,38] that there is a single accessible cysteine residue in SCP-2. Collectively, the properties described for bSCP-2 confirmed that it was the protein we expected.

Transfer ability of bSCP-2 compared with SCP-2

The ability of bSCP-2 to facilitate cholesterol transfer between membranes was examined, using [^{14}C]cholesterol-containing SUVs as donors and erythrocyte ghosts as acceptors. The ghosts were in 10-fold lipid molar excess over the SUVs and, thus, contained most of the [^{14}C]cholesterol at equilibrium. As shown in Fig. 2, there was a slow spontaneous loss of SUV cholesterol during incubation in the absence of transfer protein. Inclusion of SCP-2 (7.5 μM) in the reaction mixture resulted in a much faster analyte decay, the apparent first-order rate constant being 0.12 min^{-1} , i.e. ~ 100 -fold greater than that for spontaneous decay. The kinetics of cholesterol loss from SUVs were identical to those of uptake by ghosts (not shown), indicating that these effects can be completely accounted for by translocation. bSCP-2 also accelerated cholesterol transfer (Fig. 2), the rate constant in this case (0.032 min^{-1}) being $\sim 1/4$ that determined for SCP-2 at the same concentration. Thus, bSCP-2 was found to be functional as a transfer protein, but its efficiency was less than that of unmodified SCP-2. Defatted BSA, even at 7-fold higher molar concentration, barely enhanced the transfer rate (Fig. 2), indicating that the effects described were highly specific to bSCP-2 and SCP-2.

Optimal conditions for SPR

For valid SPR determinations, it was essential that concentrations of lipid analytes be known with high accuracy. It was apparent in initial experiments that significant amounts of any given analyte, e.g. linoleate, were adhering to the walls of polypropylene tubes in which solutions were prepared. We attempted to correct this by introducing a detergent (e.g. SDS, P20, or CHAPS) at a sufficiently low level so as not to interfere with SPR measurements. SDS and P20 in concentrations as low as 0.005 % (w/v) gave significant bSCP-2 binding signals on their own (not shown) and, thus, were unsuitable. SDS (0.02%) had initially been used to strip lipids from bSCP-2 between SPR runs. However, even after multiple cleaning steps, a significant amount of it remaining in the injection system interacted with bSCP-2, producing a high background signal from pure buffer injections (Fig. 3A). By contrast, nonionic CHAPS (CMC $\sim 9 \text{ mM}$) gave no significant binding response when injected in concentrations up to at least 0.005% (80 μM) (Fig. 3B). At 0.4% (6.5 mM), CHAPS gave a binding response of ~ 75 units (Fig. 3B), which was 10-times lower than that produced by SDS at 1/10 this concentration, i.e. 0.02 % (0.69 mM) (Fig. 3A). Thus, CHAPS binding by bSCP-2 was substantially weaker than SDS binding. Moreover, no significant binding of residual CHAPS was observed during a follow-up buffer injection (Fig. 3B). Therefore, we used CHAPS (0.4%) throughout for bSCP-2 regeneration. An additional advantage of CHAPS is that it minimized lipid loss via adsorption to contact surfaces. For this purpose, we included 0.001% (16 μM) CHAPS in all SPR running buffers, except where indicated.

Fatty acid interactions with bSCP-2

SPR sensorgrams for linoleate association with and dissociation from immobilized bSCP-2 are shown in Fig. 4, where the fatty acid concentration was varied from 2.6 to 13.1 μM . Regeneration of available bSCP-2 between runs was accomplished by flowing 0.4% (w/v) CHAPS over the chip surface, followed by running buffer alone to bring CHAPS back to its low basal level (0.001% w/v). A constant starting baseline was observed using this protocol and reproducibility between runs was excellent (Fig. 4). Using Biacore software and a Langmuir 1:1 (mol/mol) binding model, which describes a simple reversible interaction between two molecules, we determined the on-rate constant (k_a) for linoleate to be 1330 $\text{M}^{-1}\text{s}^{-1}$ and the off-rate constant (k_d) 0.0027 s^{-1} (Table 1). The dissociation equilibrium constant (K_d) obtained from these values is 2.1 μM (Table 1).

Sensorgrams for oleate interaction with bSCP-2 are shown in Fig. 5. As in the case of linoleate, replicate runs gave nearly the same responses, and binding appeared to be much more rapid than release. Oleate's k_a , k_d , and K_d values were found to be 20600 $\text{M}^{-1}\text{s}^{-1}$, 0.005 s^{-1} , and 0.22 μM , respectively (Table 1). As can be seen, k_a and k_d for oleate are ~16-times and ~2-times greater, respectively, than the corresponding values for linoleate. Oleate's K_d is ~1/9 linoleate's, indicating that the monoenoic fatty acid associated much more avidly with bSCP-2 than the dienoic.

Cholesterol and NBD-cholesterol interactions with bSCP-2

We next examined the interaction of cholesterol and NBD-derivatized cholesterol with immobilized bSCP-2. SPR kinetic scans for natural cholesterol injected over the 0.13–0.23 μM range in running buffer are shown in Fig. 6A. Using the same stoichiometric model for curve fitting as applied to the fatty acids, we determined that the k_a and k_d values for cholesterol were 1240 $\text{M}^{-1}\text{s}^{-1}$ and $3.2 \times 10^{-4} \text{s}^{-1}$, respectively, giving a K_d of 28 nM (Table 1). This K_d , which is ~8-fold lower than that for oleate and ~75-fold lower than that for linoleate, is reflected mainly in the very low off-rate for cholesterol. Our purpose for examining NBD-cholesterol binding by SPR was twofold: (i) as a means of validating our SPR data for SA-tethered bSCP-2, assuming that a comparable K_d could be obtained for solution-based SCP-2 using a different approach (fluorescence titration); and (ii) to directly assess what effects, if any, the NBD label has on cholesterol's binding characteristics. SPR sensorgrams for NBD-cholesterol over the 0.4–0.7 μM range are shown in Fig. 6B with the corresponding kinetic constants and equilibrium constant in Table 1. As can be seen, the k_a and k_d values for NBD-cholesterol are ~90-fold and ~125-fold greater, respectively, than those for unmodified cholesterol. On the other hand, the K_d values differ by only ~35% (28 nM for cholesterol vs. 38 nM for NBD-cholesterol). The higher K_d for NBD-cholesterol suggests that the fluorophore group does weaken binding, but not grossly so, at least for this particular lipid. Fluorescence titration of SCP-2 with NBD-cholesterol under conditions identical to those used for SPR analysis (Fig. 6B) gave the binding response curve shown in Fig. 7. A reciprocal plot of the data ($1/F$ vs. $1/[\text{analyte}]$) was found to be linear throughout the NBD-cholesterol concentration range used (Fig. 7, inset), exhibiting a K_d of $31.7 \pm 6.0 \text{ nM}$, which differs from that determined by SPR (Table 1) by only ~16%. The quite close agreement of these values, obtained by two independent physical methods, instills confidence that the SPR approach gave valid kinetic and equilibrium binding data.

DISCUSSION

Isolated naturally occurring or recombinant SCP-2 is known to interact with a wide variety of lipids, including fatty acids, fatty acyl-CoAs, phospholipids, and sterols [7,8]. Heretofore, these interactions have typically been studied using lipids conjugated with some type of relatively polar reporter group [7,16,17]. Such groups not only facilitated the measurement of binding,

but, in some cases, also increased the aqueous solubility of the lipid ligand and reduced any tendency to adsorb to container walls. Lipids conjugated with fluorophores such as pyrene and NBD have been commonly used for studying binding to SCP-2 and other lipid transfer proteins [16,17,39], NBD derivatives being more popular for various reasons, including smaller size of the fluorophore group (108 Da for NBD vs. 202 Da for pyrene). Clearly, however, NBD would add significantly to the size of any given lipid of interest, increasing the mass of stearic acid, for example, by ~40% and cholesterol by ~30%. Increased bulk as well as where the fluorophore is positioned could strongly influence a lipid's binding properties. As recently pointed out for cholesterol [40,41], NBD derivatization could also affect how a lipid analyte is distributed within cells. Based on these considerations, the desirability of employing unmodified lipids for binding or transfer-distribution studies is obvious. In the case of cholesterol, one approach for accomplishing this was to employ a close structural analogue, dehydroergosterol [42], using the tetraenoic sterol's endogenous fluorescence to monitor binding by SCP-2. Although binding constants were reported [42], measurements would have been difficult due to solubility limitations of the analyte and its relatively weak fluorescence at the required working levels, i.e. below the CMC. In another approach, radiolabeled cholesterol or fatty acids were used and degree of binding to SCP-2 was assessed after removing unbound lipid with Lipidex-1000, a hydrophobic resin [42,43]. However, low analyte solubility and detection difficulties at concentrations below the CMC also affect this approach, and there is an added concern that binding equilibria may be disrupted by the Lipidex-1000 treatment. The binding properties of at least one amphiphilic ligand, 1-*O*-decanyl- β -D-glucoside have been studied by means of isothermal titration calorimetry [44]. Although this technique can be highly effective for non-lipid binding systems, determinations with natural lipids such as cholesterol and oleate would be difficult, if not impossible, due to insufficient detection sensitivity at the very low (sub-micellar) lipid concentrations required. The Biacore/SPR-based approach that we describe has several advantages over these other methods, including (i) very high sensitivity (<1 nM limit for small analytes); (ii) non-requirement for radiolabeling or attachment of reporter groups; and (iii) ability to provide not only equilibrium data (binding or dissociation constants), but also kinetic parameters (on- and off-rate constants).

The first commercially available instruments for SPR experimentation were introduced by Biacore AB *ca.* 1990 [18,19]. Since then, there has been a steadily growing interest in using this powerful technique for real time determination of rate and affinity constants for the interaction of different biomolecules, e.g. enzymes with substrate analogues or inhibitors, antibodies with antigens, and receptors with agonists or antagonists. The versatility of this methodology is documented by its wide range of applications, each dependent on attachment of one of the interacting molecules to a biosensor chip [19,20]. Optimal accessibility of ligand molecules requires that the tethered molecules not be packed too tightly or impeded from folding motions that accompany binding. The validity of the SPR approach has been rigorously established. One study in this regard [45] showed that the kinetic, equilibrium, and thermodynamic constants determined for the interaction of tethered carbonic anhydrase II with arylsulfonamide compounds closely matched those obtained using two solution-based biophysical methods, isothermal titration calorimetry and stopped flow fluorescence.

The Biacore 3000 instrument used in the present study has a low mass detection limit of ~180 Da [18]. Since this is close to the size range of the lipids of interest (fatty acids ~280 Da; cholesterol ~390 Da), we initially considered tethering the lipid ligand to a sensor chip via some linker extension and flowing SCP-2 over this surface for binding measurements. This approach has at least two advantages: markedly increased SPR sensitivity due to high protein mass, and ability to study SCP-2 in a non-modified state. However, we decided against it because of concerns about the protein's ability to recognize and access lipid ligands immobilized on a chip surface. We settled instead on the opposite arrangement in which the site-specifically modified protein (bSCP-2) was attached to a streptavidin-coated chip and

lipids were flowed across. To compensate for possible sensitivity problems due to low analyte mass, we applied near-saturating amounts of bSCP-2 to the SA chip and used flow rates that minimize mass transport effects [34]. Regeneration of the bSCP-2 sensor between runs proved to be problematic in the early stages of this work. Typically, regeneration can be accomplished by changing the pH or ionic strength of the running buffer. However, these measures proved to be inadequate for stripping residual lipid from bSCP-2, so we opted for low (< CMC) concentrations of certain detergents. SDS (0.02% w/v) effectively removed all lipid from bSCP-2, as indicated by a return to the original baseline (not shown). However, a small amount of SDS adhering to the injection needle and internal tubing continued to leach into the system, so that even after several consecutive buffer injections and washing steps, a significant binding response (~ 8 RU) was observed, presumably due to SDS interaction with bSCP-2 (Fig. 3A). Since this could interfere with lipid responses, we sought a better regeneration detergent. Among those tested, non-ionic CHAPS (0.4%, w/v) worked best and was selected for routine use. CHAPS very efficiently removed lipids from SA-tethered bSCP-2 without itself binding to the protein, nor did it produce any residual responses as observed with SDS (Fig. 3B). The low level of CHAPS (0.001%, w/v) used in running buffers also proved important for minimizing lipid losses on plastic tubing and other surfaces. In addition, EDTA and DFO were included to prevent any copper- or iron-catalyzed oxidation of bSCP-2 or lipid analytes [14].

Using the indicated running buffer for SPR analyses, we determined the kinetic and equilibrium constants for bSCP-2 interaction with two unsaturated fatty acids, linoleate and oleate. Neither of these (even as an NBD derivative) has been previously examined for its ability to interact with SCP-2, the only C18 fatty acid so studied being NBD-stearate [7]. Binding of stearate itself to bSCP-2 was not examined due to this fatty acid's extremely low aqueous solubility. Linoleate and oleate were studied at concentrations well below their respective CMC levels of ~150 μ M [29] and ~15 μ M [30] at pH 7.4 and 25 °C. We found that there was a striking difference in the binding kinetics of these ligands, oleate having a 15-fold greater k_a and a 2-fold greater k_d , giving a dissociation equilibrium constant (K_d) 9-times lower than that of linoleate. Monoenoic oleate is less hydrophilic than dienoic linoleate and this could possibly explain its higher on-rate and lower K_d value, given that fatty acid binding occurs in a relatively hydrophobic pocket of SCP-2 [8]. It is worth noting that the K_d we determined for oleate (~220 nM) is within range of the values previously reported for fluorophore-labeled stearate, e.g. 230 nM for NBD-stearate and 260 nM for rhodamine B-stearate [7], suggesting that the labeling groups did not grossly perturb binding, at least for a C18 fatty acid.

In the case of cholesterol, it was necessary to work at concentrations above its reported aqueous CMC of 30–40 nM [46] in order to detect SPR responses (Fig. 6A). However, the regularity of the SPR profiles, uniformly rising with increasing sterol concentration, suggests that this analyte was predominantly monomeric during the analyses. We attribute this to the presence of CHAPS in the running buffer, although the molecular basis for this is not clear. It is also not clear why the k_a and k_d values for cholesterol were so much lower than those of NBD-cholesterol, which had the highest k_a among the ligands examined (Table 1). On the other hand, cholesterol had the lowest k_d by far, suggesting a relatively high activation energy for departure. Cholesterol had to be studied over a very narrow concentration range, limited by its very low solubility on the one hand and SPR sensitivity on the other. Three different cholesterol concentrations gave three distinguishable and reproducible SPR responses. According to a recent report [47], wide concentration ranges (e.g. 100-fold) are not always necessary for SPR analyses of small molecule-protein interactions; reliable kinetic constants can be determined using as few as two different analyte concentrations. The k_a and k_d values of unmodified cholesterol differed substantially from those of NBD-cholesterol (Table 1), yet the K_d of cholesterol was only ~30% lower than that of NBD-cholesterol. This suggests that the binding equilibrium was, in fact, affected by the NBD moiety, but not markedly so.

A key finding of this study was that K_d for the interaction of NBD-cholesterol with SCP-2 in solution, as determined by fluorescence titration, was similar to that determined by SPR for bSCP-2 tethered to a SA chip. The fact that the latter value could be nearly duplicated by an independent technique clearly establishes the validity of the SPR approach we describe. Of added importance, one can deduce from this finding that in terms of lipid binding competency, tethered bSCP-2 was configured similarly to SCP-2 in homogeneous solution. If this were so, one might ask why bSCP-2 was less effective in translocating cholesterol between membranes than SCP-2 (Fig. 2). The biotinylated AviTag (~3.5 kDa) could make bSCP-2's conformation in solution quite different from that of SCP-2, and folding of the peptide extension might interfere with membrane targeting or lipid binding, possibly explaining the diminished translocation. Also, the estimated isoelectric point of bSCP-2 is significantly lower than that of SCP-2 (pI ~8.1 vs. 9.2) and this might also affect the transfer ability of the former. On the other hand, bSCP-2 attachment to a SA chip via the AviTag could prevent any folding interference, thus tending to "normalize" the protein with regard to lipid binding. Studies dealing with these questions are currently underway. It is important to point out that NBD-cholesterol binding by SCP-2 in solution was studied previously by others using fluorescence titrimetry, K_d values of 4 nM [39] and 112 nM [17] being reported. These are significantly different from our values, one possible explanation being that different incubation conditions were used (temperature, pH, and type of buffer).

The SPR approach we describe is entirely novel for the general purpose of studying interactions between unmodified natural lipids and lipid transfer proteins. Only one other study is known in which a biotinylated AviTag-like extension was used to immobilize a protein for SPR analysis [48]. However, this involved a protein-protein interaction system for which only qualitative effects of mutagenesis (not kinetic and equilibrium constants) were described. Of added note is another recent study describing the SPR behavior of the plasma phospholipid transfer protein (PLTP) [49]. Once again, however, only protein-protein (PLTP-apolipoprotein) interactions were investigated, not lipid-protein interactions, so the present study is entirely original in this respect. For quantitative investigations of lipid-protein interactions, the advantages of being able to use non-labeled lipids and to assess both binding and dissociation kinetics as well as equilibrium constants make our approach very attractive compared with other existing methods. The full spate of information obtained via SPR measurements could prove invaluable in understanding action mechanisms. We have used SCP-2, a ubiquitous lipid binding/translocating protein, as the prototype for developing the SPR-based approach. There are numerous other intracellular and extracellular lipid interacting proteins that should be amenable to this approach after being appropriately engineered (e.g. biotinylated) for biosensor attachment. Included among these are the intracellular PC and PI transfer proteins, fatty acid binding proteins (FABPs), steroidogenic acute regulatory protein (StAR), oxysterol binding proteins, and plasma lipid transfer proteins. For many of these, lipid interactions have not yet been examined rigorously, and for others the data are limited (e.g. K_d estimates only) and/or may be questionable because lipids with large reporter groups were used.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Zilversmit DB. Lipid transfer proteins. *J Lipid Res* 1984;25:1563–1569. [PubMed: 6397561]
2. Scallen TJ, Pastuszyn A, Noland BJ, Chanderbhan R, Kharroubi A, Vahouny GV. Sterol carrier and lipid transfer proteins. *Chem Phys Lipids* 1985;38:239–261. [PubMed: 3910286]
3. Wirtz KWA. Phospholipid transfer proteins. *Annu Rev Biochem* 1991;60:73–99. [PubMed: 1883207]
4. Seedorf U, Ellinghaus P, Nofer JR. Sterol carrier protein-2. *Biochim Biophys Acta* 2000;1486:45–54. [PubMed: 10856712]
5. Tall A. Plasma lipid transfer proteins. *Annu Rev Biochem* 1995;64:235–257. [PubMed: 7574481]
6. Stein O, Stein Y. Lipid transfer proteins (LTP) and atherosclerosis. *Atherosclerosis* 2005;178:217–230. [PubMed: 15694928]
7. Gallegos AM, Atshaves BP, Storey SM, Starodub O, Petrescu AD, Huang H, McIntosh AL, Martin GG, Chao H, Kier AB, Schroeder F. Gene structure, intracellular localization, and functional roles of sterol carrier protein-2. *Prog Lipid Res* 2001;40:498–563. [PubMed: 11591437]
8. Stolowich NJ, Petrescu AD, Huang H, Martin GG, Scott AI, Schroeder F. Sterol carrier protein-2: structure reveals function. *Cell Mol Life Sci* 2002;59:193–212. [PubMed: 11915938]
9. Keller GA, Scallen TJ, Clarke D, Maher PA, Krisans SK, Singer SJ. Subcellular localization of sterol carrier protein-2 in rat hepatocytes: its primary localization in peroxisomes. *J Cell Biol* 1989;108:1353–1351. [PubMed: 2925789]
10. Chanderbhan R, Noland BJ, Scallen TJ, Vahouny GV. Sterol carrier protein-2: delivery of cholesterol from adrenal lipid droplets to mitochondria for pregnenolone synthesis. *J Biol Chem* 1982;257:8928–8934. [PubMed: 7096342]
11. Billheimer JT, Gaylor JL. Effect of lipid composition on the transfer of sterols mediated by non-specific lipid transfer protein (sterol carrier protein-2). *Biochim Biophys Acta* 1990;1046:136–143. [PubMed: 2171663]
12. Gadella TWJ, Wirtz KWA. Phospholipid binding and transfer by the non-specific lipid transfer protein (sterol carrier protein-2): a kinetic model. *Eur J Biochem* 1994;220:1019–1028. [PubMed: 8143718]
13. Frolov A, Woodford JK, Murphy EJ, Billheimer JT, Schroeder F. Fibroblast membrane sterol kinetic domains: modulation by sterol carrier protein-2 and liver fatty acid binding protein. *J Lipid Res* 1996;37:1862–1874. [PubMed: 8895052]
14. Vila A, Levchenko VV, Korytowski W, Girotti AW. Sterol carrier protein-2-facilitated intermembrane transfer of cholesterol- and phospholipid-derived hydroperoxides. *Biochemistry* 2004;43:12592–12605. [PubMed: 15449949]
15. Kriska T, Levchenko VV, Korytowski W, Atshaves BP, Schroeder F, Girotti AW. Intracellular dissemination of peroxidative stress: internalization, transport, and lethal targeting of a cholesterol hydroperoxide species by sterol carrier protein-2-overexpressing hepatoma cells. *J Biol Chem* 2006;281:23643–23651. [PubMed: 16772292]
16. Nichols JW. Binding of fluorescent-labeled phosphatidylcholine to rat liver non-specific lipid transfer protein. *J Biol Chem* 1987;262:14172–14177. [PubMed: 3654657]
17. Avdulov NA, Chochina SV, Igbavboa U, Warden CS, Schroeder F, Wood WG. Lipid binding to sterol carrier protein-2 is inhibited by ethanol. *Biochim Biophys Acta* 1999;1437:37–45. [PubMed: 9931423]
18. Morton TA, Myszka DG. Kinetic analysis of macromolecular interactions using surface plasmon resonance biosensors. *Methods Enzymol* 1998;295:268–282. [PubMed: 9750223]
19. Myszka DG. Kinetic, equilibrium, and thermodynamic analysis of macromolecular interactions with BIACORE. *Methods Enzymol* 2000;323:325–340. [PubMed: 10944758]
20. Karlsson R. SPR for molecular interaction analysis: a review of emerging application areas. *J Mol Recogn* 2004;17:151–161.
21. Matsuura JE, George HJ, Ramachandran N, Alvarez JG, Strauss JF, Billheimer JT. Expression of the mature and the pro-form of human sterol carrier protein-2 in *Escherichia coli* alters bacterial lipids. *Biochemistry* 1993;32:567–572. [PubMed: 8422367]
22. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976;72:248–254. [PubMed: 942051]

23. Chattopadhyay MK, Tabor CW, Tabor H. Spermidine but not spermine is essential for hypusine biosynthesis and growth in *Saccharomyces cerevisiae*: spermine is converted to spermidine *in vivo* by the FMS1-amine oxidase. *Proc Natl Acad Sci USA* 2003;100:13869–13874. [PubMed: 14617780]
24. Yamamoto R, Kallen CB, Babalola GO, Rennert H, Billheimer JT, Strauss JF. Cloning and expression of a cDNA encoding human sterol carrier protein-2. *Proc Natl Acad Sci USA* 1991;88:463–467. [PubMed: 1703300]
25. Cull MG, Schatz PJ. Biotinylation of proteins *in vivo* and *in vitro* using small peptide tags. *Methods Enzymol* 2000;326:430–440. [PubMed: 11036656]
26. Laitinen OH, Nordlund HR, Hytönen VP, Uotila STH, Marttila AT, Savolainen J, Airenne KJ, Livnah O, Bayer EA, Wilchek M, Kulomaa MS. Rational design of an active avidin monomer. *J Biol Chem* 2003;278:4010–4014. [PubMed: 12458212]
27. Vila A, Korytowski W, Girotti AW. Dissemination of peroxidative stress via intermembrane transfer of lipid hydroperoxides: model studies with cholesterol hydroperoxides. *Arch Biochem Biophys* 2000;380:208–218. [PubMed: 10900151]
28. Vila A, Korytowski W, Girotti AW. Spontaneous intermembrane transfer of cholesterol-derived hydroperoxide species: kinetic studies with model membranes and cells. *Biochemistry* 2001;40:14715–14726. [PubMed: 11724586]
29. Gietzen K, Xu YH, Galla HJ, Bader H. Multimers of anionic amphiphiles mimic calmodulin stimulation of cyclic nucleotide phosphodiesterase. *Biochem J* 1982;207:637–640. [PubMed: 6299276]
30. Lagocki JW, Emken EA, Law JH, Kezdy FJ. Kinetic analysis of the action of soybean lipoxygenase on linoleic acid. *J Biol Chem* 1976;251:6001–6006. [PubMed: 823156]
31. Chattopadhyay A, Mukherjee S. Red edge excitation shift of a deeply embedded membrane probe: implications in water penetration in the bilayer. *J Phys Chem B* 1999;103:8180–8185.
32. Richieri GV, Ogata RT, Kleinfeld AM. The measurement of free fatty acid concentration with the fluorescent probe ADIFAB: a practical guide for the use of the ADIFAB probe. *Mol Cell Biochem* 1999;192:87–94. [PubMed: 10331662]
33. Information provided by the ADIFAB2 kit supplier, FFA Sciences.
34. Myszka DG, Morton TA, Doyle ML, Chaiken IM. Kinetic analysis of a protein antigen-antibody interaction limited by mass transport on an optical biosensor. *Biophys Chem* 1997;64:127–37. [PubMed: 9127943]
35. Cannon MJ, Papalia GA, Navratilova I, Fisher RJ, Roberts LR, Worthy KM, Stephen AG, Marchesini GR, Collins EJ, Casper D, Qiu H, Satpaev D, Liparoto SF, Rice DA, Gorshkova II, Darling RJ, Bennett DB, Sekar M, Hommema E, Liang AM, Day ES, Inman J, Karlicek SM, Ullrich SJ, Hodges D, Chu T, Sullivan E, Simpson J, Rafique A, Luginbuhl B, Westin SN, Bynum M, Cachia P, Li YJ, Kao D, Neurauter A, Wong M, Swanson M, Myszka DG. Comparative analyses of a small molecule/enzyme interactions by multiple users of Biacore technology. *Anal Biochem* 2004;330:98–113. [PubMed: 15183767]
36. Hirel PHH, Schmitter JM, Dessen P, Fayat G, Blanquet S. Extent of N-terminal methionine excision from *E. coli* proteins is governed by the side-chain length of the penultimate amino acid. *Proc Natl Acad Sci USA* 1989;86:8247–8251. [PubMed: 2682640]
37. Ellman GL. Tissue sulfhydryl groups. *Arch Biochem Biophys* 1959;82:70–77. [PubMed: 13650640]
38. Westerman J, Wirtz KWA. The primary structure of the non-specific lipid transfer protein (sterol carrier protein-2) from bovine liver. *Biochem Biophys Res Commun* 1985;127:333–338. [PubMed: 3977925]
39. Stolowich NA, Frolov A, Petrescu AD, Scott AI, Billheimer JT, Schroeder F. Holo-sterol carrier protein-2: 13C-NMR investigation of cholesterol and fatty acid binding sites. *Biochemistry* 1999;274:35425–35433.
40. Mukherjee S, Zha X, Tabas I, Maxfield FR. Cholesterol distribution in living cells: fluorescence imaging using dehydroergosterol as a fluorescent cholesterol analog. *Biophys J* 1998;75:1915–1925. [PubMed: 9746532]
41. Maxfield FR, Wustner D. Intracellular cholesterol transport. *J Clin Invest* 2002;110:891–898. [PubMed: 12370264]

42. Colles SM, Woodford JK, Moncecchi D, Myers-Payne SC, McLean LR, Billheimer JT, Schroeder F. Cholesterol interaction with recombinant human sterol carrier protein-2. *Lipids* 1995;30:795–803. [PubMed: 8577222]
43. Vork MM, Glatz JF, Surtel DA, van der Vusse GJ. Assay of the binding of fatty acids by proteins: evaluation of the Lipidex 1000 procedure. *Mol Cell Biochem* 1990;98:111–117. [PubMed: 1702508]
44. Jatzke C, Hinz HJ, Seedorf U, Assmann G. Stability and binding properties of wild-type and C17S mutated human sterol carrier protein-2. *Biochim Biophys Acta* 1999;1432:265–274. [PubMed: 10407148]
45. Day YSN, Baird CL, Rich RL, Myska DG. Direct comparison of binding equilibrium, thermodynamic, and rate constants determined by surface- and solution-based biophysical methods. *Protein Science* 2002;11:1017–1025. [PubMed: 11967359]
46. Haberland ME, Reynolds JE. Self-association of cholesterol in aqueous solution. *Proc Natl Acad Sci USA* 1973;70:2313–2316. [PubMed: 4525165]
47. Onell A, Andersson K. Kinetic determinations of molecular interactions using Biacore: minimum data requirements for efficient experimental design. *J Mol Recognit* 2005;18:307–317. [PubMed: 15952219]
48. Slep KC, Rogers SL, Elliott SL, Ohkura H, Kolodziej PA, Vale RD. Structural determinants for EB1-mediated recruitment of APC and spectraplakins to the microtubule plus end. *J Cell Biol* 2005;168:587–598. [PubMed: 15699215]
49. Janis MT, Metso J, Lankinen H, Strandin T, Olkkonen W, Rye KA, Jauhiainen M, Ehnholm C. Apolipoprotein E activates the low-activity form of human phospholipid transfer protein. *Biochem Biophys Res Commun* 2005;331:333–340. [PubMed: 15845396]

Abbreviations

BSA	bovine serum albumin
bSCP-2	biotinylated SCP-2
CHAPS	3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate hydrate
CMC	critical micelle concentration
DCP	dicetylphosphate
DFO	desferrioxamine
HEPES	4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid
IPTG	isopropyl β -D-thiogalactoside
NBD	<i>N</i> -(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino
NBD-cholesterol	22-[<i>N</i> -(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-23,24-bisnor-5-cholen-3 β -ol
PC	phosphatidylcholine

PBS	Chelex-treated phosphate-buffered saline (25 mM sodium phosphate, 125 mM NaCl, pH 7.4)
RU	resonance unit
SCP-2	sterol carrier protein-2
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SPR	surface plasmon resonance
SUV(s)	small unilamellar vesicle(s)

Figure 1.

Electrophoretic properties of purified bSCP-2 and SCP-2. Proteins were dissolved in NuPAGE LDS sample buffer under reducing conditions and heated to 70 °C before loading onto a NuPAGE 4–12% acrylamide/bis-acrylamide gel. Protein bands were visualized by SimplyBlue staining. Analyte lanes are as follows: (1) Molecular mass (kDa) standards, (2) crude lysate of bSCP-2-expressing BL21(DE3) cells; (3) purified bSCP-2; (4) purified SCP-2. Protein loaded: 2.9 µg (lane 3); 3.5 µg (lane 4).

Figure 2.

Comparative abilities of bSCP-2 and SCP-2 to facilitate cholesterol transfer between membranes. Reaction mixtures containing egg PC/[^{14}C]cholesterol/DCP (100:80:2 by mol) SUV donors (0.06 mM lipid) and red cell ghost acceptors (0.6 mM lipid) either lacked proteins (○) or contained 7.5 μM bSCP-2 (●), 7.5 μM SCP-2 (▲), or 50 μM BSA (□) in PBS/0.1 mM DFO/0.1 mM EDTA (pH 7.4). At various times during incubation at 37 °C, samples were centrifuged and SUV-containing supernatants were extracted. Recovered lipid fractions were analyzed for [^{14}C]cholesterol content by scintillation counting. First-order plots for cholesterol loss from SUVs over time are shown. Means $\pm$ deviation of values from duplicate experiments are shown.

Figure 3.

Sensorgrams comparing abilities of SDS and CHAPS to interact with immobilized bSCP-2. The running buffer in each case contained 10 mM HEPES and 25 μ M each of DFO and EDTA (pH 7.4). (A) SDS responses. Sequential injections were as follows: buffer alone (a), 0.02% (w/v) SDS in buffer (b), and then buffer alone (c, d, e). Injection periods were as follows: 2 min (a, c, d, e); 3 min (b). (B) CHAPS responses. Sequential injections were as follows: buffer alone (a) followed by 0.001%, 0.005%, and 0.4% (w/v) CHAPS in buffer (b, c, and d, respectively), then buffer alone (e). Time period for each injection was 2 min. Pure running buffer was introduced at the end of each injection period in (A) and (B).

Figure 4.

Sensorgrams for linoleic acid interaction with immobilized bSCP-2. Sample injection rate was 50 $\mu\text{l}/\text{min}$ and operating temperature was 25 $^{\circ}\text{C}$. A duplicate series of injections at 2.6, 5.2, 7.9, 10.5, and 13.1 μM linoleate (reading from the bottom to top scan) is represented, as denoted by (a) and (b). Each concentration was examined twice in succession, (a) then (b), beginning with the highest and ending with the lowest. Upward arrows mark points of fatty acid solution injection after baseline was established; downward arrows mark ends of solution injection and beginning of pure buffer flow. Scans were globally fit (thin black lines) to a 1:1 (mol/mol) interaction model, using a floating term to correct for bulk refractive index changes. Average χ^2 value (closeness of fit) is 0.08.

Figure 5. Sensorgrams for oleic acid interaction with immobilized bSCP-2. A duplicate series of injections, (a) and (b), at 5.0, 5.8, 6.7, 7.5, and 8.3 μM oleate (reading from the bottom to top scan) is represented. Other details were as described in Fig. 4. Average χ^2 value is 0.11.

Figure 6.

Sensorgrams depicting interaction of cholesterol and NBD-cholesterol with immobilized bSCP-2. (A) *Cholesterol*. Injected concentrations were as follows: 0.13 μM ; 0.17 μM ; 0.23 μM (reading from bottom to top scan). (B) *NBD-cholesterol*. Injected concentrations were: 0.41, 0.48, 0.54, 0.61, and 0.68 μM (reading from bottom to top scan). One scan representative of two at each concentration is shown in (A) and (B). The data were fit to 1:1 (mol/mol) interaction model with a floating term to correct for bulk refractive index changes. Average χ^2 value is 0.10 for cholesterol and 0.18 for NBD-cholesterol.

Figure 7.

Fluorescence titration of SCP-2 with NBD-cholesterol. SCP-2 (400 nM) in running buffer at 25 °C was titrated with NBD-cholesterol (2.0 μ M in DMF). A reference titration without SCP-2 was run alongside. F in arbitrary units represents the corrected emission peak area (reaction mixture – reference). Plotted data ($\circ$) show the binding isotherm for NBD-cholesterol. Means $\pm$ SE of values from three separate experiments are represented. The *inset* shows a reciprocal plot of the data, where [L] represents the concentration of free NBD-cholesterol. The K_d determined from this plot is 31.7 ± 6.0 nM.

Table 1SPR-assessed on/off rate constants and equilibrium constants for various analytes^a

Analyte	$k_a \times 10^{-3} \text{ (M}^{-1}\text{s}^{-1}\text{)}$	$k_d \times 10^3 \text{ (s}^{-1}\text{)}$	$K_d \text{ (nM)}$
Linoleic Acid	1.33 ± 0.11	2.71 ± 0.05	2060 ± 170
Oleic Acid	20.6 ± 1.0	4.59 ± 0.06	222 ± 12
Cholesterol	1.24 ± 0.57	0.034 ± 0.02	27.6 ± 20.0
NBD-cholesterol	111 ± 3.3	4.27 ± 0.04	37.7 ± 1.0

^aMean values from duplicate experiments are shown, as represented in Fig. 4 (linoleic acid), Fig. 5 (oleic acid), Fig. 6A (cholesterol), and Fig. 4B (NBD-cholesterol). k_a , association rate constant; k_d , dissociation rate constant; K_d , equilibrium dissociation constant.