Although sterol carrier protein-2 (SCP-2) stimulates sterol transfer in vitro, almost nothing is known regarding the identity of the putative cholesterol binding site. Furthermore, the interrelationship(s) between this SCP-2 ligand binding site and the recently reported SCP-2 long chain fatty acid (LCFA) and long chain fatty acyl-CoA (LCFA-CoA) binding site(s) remains to be established. In the present work, two SCP-2 ligand binding sites were identified. First, both $[^4\text{C}]$cholesterol and $22-(\text{N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)}$)-23,24-bisnor-5-cholen-3$\beta$-ol (NBD-cholesterol) binding assays were consistent with a single cholesterol binding site in SCP-2. This ligand binding site had high affinity for NBD-cholesterol, $K_d = 4.15 \pm 0.71 \text{ nM}$. $^{13}\text{C}$ NMR-labeled ligand competition studies demonstrated that the SCP-2 high affinity cholesterol binding site also bound LCFA-CoA. However, only the LCFA-CoA was able to effectively displace the SCP-2-bound $[^4\text{C}]$cholesterol. Thus, the ligand affinities at this SCP-2 binding site were in the relative order cholesterol $=$ LCFA-CoA $>$ LCFA. Second, $^{13}\text{C}$ NMR studies demonstrated the presence of another ligand binding site on SCP-2 that bound either LCFA or LCFA-CoA but not cholesterol. Photon correlation spectroscopy was consistent with SCP-2 being monomeric in both liganded and unliganded states. In summary, both $^{13}\text{C}$ NMR and fluorescence techniques demonstrated for the first time that SCP-2 had a single high affinity binding site that bound cholesterol, LCFA, or LCFA-CoA. Furthermore, results with $^{13}\text{C}$ NMR supported the presence of a second SCP-2 ligand binding site that bound either LCFA or LCFA-CoA but not cholesterol. These data contribute to our understanding of a role for SCP-2 in both cellular cholesterol and LCFA metabolism.

Although great advances have been made in our understanding of vascular lipid transport via serum lipoproteins, much less is known regarding intracellular trafficking pathways of lipids. While spontaneous desorption and intracellular diffusion of lipids occur, they do not account for (i) the asymmetric distribution of cellular lipids, (ii) the synthesis of cholesterol in and targeted efflux from the relatively cholesterol poor endoplasmic reticulum, (iii) the lysosomal release and intracellular targeting of cholesterol, fatty acids, and glycerides, or (iv) high density lipoprotein-mediated reverse cholesterol transport. Cellular lipids are transferred and targeted within the cell via vesicular pathways, cytosolic proteins, and possibly via as yet undefined interactions between cytosolic proteins and vesicular pathways.

In addition to vesicular pathways, intracellular cholesterol is believed to be transferred and targeted within the cell via proteins such as SCP-2$^1$ (reviewed in Refs. 1–5), caveolin (reviewed in Refs. 4 and 6–11), or steroidogenic acute regulatory protein (12). SCP-2 is primarily a soluble protein, caveolin exists in both membrane-bound and soluble homo- and hetero-complex forms, and steroidogenic acute regulatory protein traffics from the endoplasmic reticulum to the inner mitochondrial membrane by mechanism(s) not yet understood.

A variety of studies show positive correlation between SCP-2 expression and intracellular cholesterol transfer in vivo: biliary cholesterol secretion (13–15), lung surfactant formation (16), intestinal cholesterol absorption (17, 18), macrophage foam cell formation (19), diabetes (20), and cholesterol oxidation (21–23). Likewise, studies with intact transfected cells overexpressing SCP-2 confirm a role for SCP-2 in intracellular sterol trafficking (24–30). Despite these observations, very little is known regarding the mechanism whereby SCP-2 transfers cholesterol between membranes. It has been suggested that interaction of SCP-2 with cholesterol (31–34) is essential for SCP-2-mediated intermembrane sterol transfer in vitro (35). Unfortunately, clear demonstration of cholesterol binding or saturation binding of other sterols to SCP-2 has been difficult to achieve.

In addition to its involvement in intermembrane cholesterol transfer, recent reports that SCP-2 also interacts with long chain fatty acids (LCFA) and long chain fatty acyl-CoAs (LCFA-CoA) suggest additional role(s) for this protein in intracellular LCFA and/or LCFA-CoA trafficking. The pathway(s) whereby LCFA and their CoA derivatives traffic and are targeted within the cell are as yet undefined (reviewed in Refs. 36 and 37). Increasing evidence shows that the intracellular fatty acid binding proteins function in intracellular trafficking by enhancing LCFA uptake, intracellular diffusion, or metabolic targeting (reviewed in Refs. 36–39). For over a decade, it was thought that one of the intracellular lipid binding proteins, SCP-2, did not bind fatty acids in the Lipidx-1000 assay and was not involved in fatty acid metabolism (40). However, more

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$^1$ The abbreviations used are: SCP-2, sterol carrier protein-2; LCFA, long chain fatty acid(s); NBD-cholesterol, 22-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-23,24-bisnor-5-cholen-3$\beta$-ol; I- and L-FABP, intestinal and liver fatty acid-binding protein, respectively.
recent data using improved fluorescence and $^{13}$C NMR binding assays showed that the SCP-2 binds LCFA (41–43) and LCFA-CoA (44) with high affinity, $K_d$ values of 200–400 and 2–4 nm, respectively. Furthermore, SCP-2 also binds branched-chain LCFA (43, 45) and may be specifically involved in the peroxisomal oxidation of branched chain LCFA (45, 46). Thus, SCP-2 clearly exhibits saturation binding of LCFA and LCFA-CoA with high affinity. Whether this ligand binding site(s) is identical to the putative cholesterol binding site remains to be determined.

The objective of the present investigation was to characterize the interrelationships of the SCP-2 binding site(s) for LCFA, LCFA-CoA, and cholesterol. $^{13}$C and fluorescent labeled LCFA and sterols were used to show for the first time that SCP-2 has CoA (44) with high affinity, assays showed that the SCP-2 binds LCFA (41–43) and LCFA-ated dithiothreitol. $[4-^{13}$C$]$cholesterol was purchased from Isotec (Midland, MI) from Cambridge Isotope Laboratories (Andover, MA) as was perdeuterated $^{13}$C NMR of Sterol Carrier Protein-2 Ligand Binding and fluorescence techniques both revealed a high affinity site at which cholesterol, fatty acid, or fatty acyl-CoA bound. SCP-2 bound sterol at this site with $K_d = 4$ nm, near that reported for LCFA-CoA, $K_d = 2–4$ nm (44). A second site, revealed by $^{13}$C NMR but not fluorescence assays, bound only fatty acid or fatty acyl-CoA, but not cholesterol. The second site was not due to oligomerization of SCP-2 at the high protein concentrations required for $^{13}$C NMR.

**EXPERIMENTAL PROCEDURES**

**Materials**

Fully enriched $[^{13}$C$]_{10}$oleic acid and $[^{1}$C$]_{1}$oleic acid were purchased from Cambridge Isotope Laboratories (Andover, MA) as was perdeuterated dithiothreitol. $[^{13}$C$]_{1}$Cholesterol was purchased from Isotec (Midland, MI). Methyl-β-cyclohexatrien was obtained from Aldrich, and 2-hydroxypropyl-β-cyclohexatrien was from American Maize-Products Co. (Hammond, IN). 22-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-23,24-bisnor-5-cholesten-3β-ol (NBD-cholesterol) was purchased from Molecular Probes Inc. (Eugene, OR). Oleic acid, oleoyl-CoA, cholesterol, and Tris were obtained from Sigma. All other chemicals were reagent grade or better.

**Isolation of Recombinant Human SCP-2**

The recombinant human SCP-2 was prepared as described earlier (47). The concentration of SCP-2 was determined by Bradford assay (48). Most recombinant SCP-2 proteins from a variety of species reported thus far contain at least one mutation near the N terminus. Deletion of large segments, e.g., 10 or more amino acids, at the amino terminus as well as some substitutions at position 20 were able to inactive the recombinant SCP-2 as determined by large changes in circular dichroic spectra and in sterol transfer assays (49). In contrast, small differences in amino terminus amino acid sequence in the recombinant SCP-2 do not result in significantly altered function. For example, recombinant human SCP-2 and native rat liver SCP-2, which differ in their amino terminus (50), enhanced intermembrane sterol transfer almost identically (49, 51). Likewise, recombinant mouse, human, or rat SCP-2 and native rat SCP-2 (all differ in the N-terminal three amino acids) as well as mutants in the N-terminal five amino acids of human recombinant SCP-2 did not significantly differ in circular dichroic structure or a variety of functional assays such as enhancement of microsomal conversion of 7-dehydrocholesterol to cholesterol, stimulation of microsomal acyl-CoA cholesterol acyltransferase, enhancement of intermembrane sterol transfer, or stimulation of intermembrane phospholipid transfer (49–52).

**Preparation of Fatty Acid-SCP-2 and Cholesterol-SCP-2**

**Complexes for NMR**

For each sample prepared, an SCP-2 (2 mg/ml) stock solution was subjected to several buffer exchanges (20 mM KP, 4 mM perdeuterated dithiothreitol, pH 7.0, in 20% D$_2$O) through a YM 3 Ultrafiltration membrane (Amicon, Beverly, MA) before being concentrated to a final volume of 400–500 µl. The final SCP-2 concentration employed was 15–20 mg/ml (1.0–1.5 mM). The $^{13}$C NMR reference spectra of apo-SCP-2 were obtained using this solution prior to complexing with fatty acid or cholesterol. Complexes of fatty acids and SCP-2 were prepared as described earlier (42). Complexes of cholesterol and SCP-2 were prepared as follows.

**Method 1**—Method 1 involved first complexing the cholesterol with O-modified cyclodextrins as described earlier (53). Cholesterol-cyclodextrin inclusion complexes were prepared with either methyl-β-cyclohexatrien or 2-hydroxypropyl-β-cyclohexatrien as the host by mixing varying amounts of $[^{4}$$^{13}$C$]$cholesterol with a 5% (w/v) solution of the cyclohexatrien at 80 °C, followed by lyophilization. $^{13}$C NMR analysis as the lyophilized complexes indicated that the highest degree of cholesterol incorporation was achieved using 2-hydroxypropyl-β-cyclohexatrien and a cholesterol concentration of 1 mg/g of 2-hydroxypropyl-β-cyclohexatrien (data not shown). This sample was then used in attempt to deliver cholesterol to SCP-2. However, removal of the 2-hydroxypropyl-β-cyclohexatrien by membrane filtration led to a loss of cholesterol, indicating a preference for the cyclohexatrien over SCP-2.

**Method 2**—Method 2 involved the addition of aliquots (2-fold molar excess relative to SCP-2) of the $[^{4}$$^{13}$C$]$cholesterol (20 mM in CHCl/MeOH (2:1)) to a clean NMR tube and evaporation of the solvent with a stream of argon and with gentle heating (35–40 °C). SCP-2 in buffer (20 mM KP, 4 mM perdeuterated dithiothreitol, pH 7.0 in 20% D$_2$O) were added directly to the tube containing the solid cholesterol, and the tube was incubated at 37 °C for 30–60 min, being removed intermitently to be briefly sonicated. The sample was then used for NMR spectroscopy, without further removal of residual suspended cholesterol.

**Carbon-13 NMR Spectroscopy**

A Bruker ARX-500 spectrometer equipped with a 5-mm C/H probe was used to obtain proton-decoupled $^{13}$C NMR spectra at 125.76 MHz. Low power (1/4-watt) proton decoupling was accomplished using the pulse program WALTZ sequence in the Bruker software package. Conditions were as described earlier (42). The intense signal of the SCP-2 ε-Lys/β-Leu carbons was used as an internal chemical shift reference (39.45 ppm) (54).

**NBD-Cholesterol Binding Assay**

SCP-2 binding affinity for NBD-cholesterol was determined as described previously (41) with the following modifications. A 2-ml sample of 11.1 mM SCP-2 in phosphate buffer (10 mM, pH 7.4) was titrated with small increments of NBD-cholesterol (0.5–2.0 µM) dissolved in dimethylformamide. The NBD-cholesterol stock solution concentration was 14 µM. Each sample and blank (without SCP-2) were thoroughly mixed and allowed to equilibrate for 2–4 min to permit stable measurement of the fluorescent signal. NBD-cholesterol fluorescence was excited at 465 nm, and emission was recorded in the region of 500–600 nm. Excitation and emission slits were 8 and 16 nm, respectively. The integrated NBD-cholesterol emission was measured after each addition of the ligand, corrected for the background and blank, and used to construct the binding isotherm. Data were fit using a simple, single binding site model as described (41). All experiments were performed at 37 °C.

**Size Distribution of Unliganded and Liganded SCP-2**

Photon correlation spectroscopy with a Coulter N4 Plus Photon Correlation Spectrometer (Beckman-Coulter Inc., Miami, FL) was used to determine the particle size distribution of unliganded SCP-2 (1 mM), oleoyl-CoA (2 mM), and a 2:1 complex of 1 mM SCP-2 plus 2 mM oleoyl-CoA. The samples were run at the 90° angle for 900 s directly. Size distribution processor weight analysis and size distribution processor weight peak values were reported. Photon correlation spectroscopy optimally measures particle size in the range of 3–3000 nm in diameter, within the size range of SCP-2 near 4.1 nm as indicated by fluorescence spectroscopy (43, 44).

**RESULTS**

**Binding of [4-$^{13}$C$]$/Cholesterol to SCP-2—**As outlined in the Introduction, SCP-2 has been widely implicated in sterol transport, etc.; however, the direct detection of cholesterol binding to SCP-2 has been difficult due to the sterol’s insolubility in an aqueous environment, particularly at concentrations near physiological significance (reviewed in Ref. 55). Therefore, two different approaches were used to demonstrate binding of [4-$^{13}$C$]$Cholesterol to SCP-2.

First, O-modified cyclohexadextrins were used to increase the aqueous solubility of the [4-$^{13}$C$]$Cholesterol. The utility of employing O-modified cyclohexadextrins for this purpose and for increasing delivery of several sterols including cholesterol has been demonstrated (reviewed in Refs. 36 and 56). However, several attempts to utilize cholesterol-cyclohexadextrin inclusion complexes in the present study, using hydroxypropyl-β-cyclohexadextrin as the host, did not conclusively demonstrate SCP-2
binding of [4-13C]cholesterol in the presence of the cyclodextrin carrier. Furthermore, removal of the cyclodextrin carrier via membrane filtration resulted in complete loss of the [4-13C]cholesterol signal (data not shown), indicating a binding preference of [4-13C]cholesterol for cyclodextrin over SCP-2.

Second, [4-13C]cholesterol was solubilized by SCP-2 from solid [4-13C]cholesterol. Fig. 1 shows the outcome of adding a 1 mM solution of SCP-2 to an NMR tube that has been previously coated with a film of 2 eq of [4-13C]cholesterol. After incubation for 1 h at 37 °C, the 13C NMR spectrum was obtained (Fig. 1). A new resonance, not present in SCP-2 alone (Fig. 1A), was identified at δ 40.8 ppm (Fig. 1B). The chemical shift of the new peak was similar to that observed for the enriched C-4 carbon of cholesterol in CDCl3 (δ 42.7 ppm) as well as to that bound to 2-hydroxypropyl-β-cyclodextrin (δ 41.7 ppm) (Table 1). The sample was then transferred to a new NMR tube containing an additional 2 eq of solid [4-13C]cholesterol, again incubated, and the NMR spectrum was determined. A further increase in the intensity of the new peak at δ 40.8 ppm was observed (Fig. 1C), indicating SCP-2 in Fig. 1B had not yet been saturated with sterol. Subsequent attempts to bind more [4-13C]cholesterol led to no further increase in signal intensity. Since solid sterol residue was left behind after each incubation, it was difficult to ascertain the amount of cholesterol complexed to SCP-2 based only on the number of equivalents of [4-13C]cholesterol added to the NMR tube. However, judging by comparison to the peak areas observed for other 1:1 complexes of ligand to protein (42, 57), it was estimated that under these conditions SCP-2 bound a single equivalent of cholesterol.

SCP-2 Binding of NBD-cholesterol—Since it was not possible to accurately determine a $K_d$ for SCP-2 binding cholesterol from the NMR data, the binding of a fluorescent NBD-labeled cholesterol was determined. Our laboratory had previously shown that SCP-2 and another LCFA binding protein (L-FABP) did not bind the NBD group by itself, but both proteins bound NBD-LCFA with a $K_d$ value similar to that for other LCFA (41, 58). NBD-cholesterol binding to SCP-2 showed a saturation binding curve from which a $K_d$ of 4.15 ± 0.71 nM (n = 4) was derived. The affinity of SCP-2 for NBD-cholesterol was at least 50-fold higher than that for LCFA (41–43) but remarkably similar to that for LCFA-CoA binding to SCP-2, $K_d$ = 2.8–4.5 nM, depending on the structure of the LCFA-CoA (44).

13C Chemical Shifts of SCP-2 Bound Versus Unbound Fully Enriched [13C18]Oleate—Prior to using 13C-labeled fatty acids to determine if the cholesterol and fatty acid binding sites were the same or different, it was necessary to first characterize the interaction of fatty acid with SCP-2. Our laboratory previously showed 13C NMR spectra of single labeled fatty acid species, 13C-labeled at the C-1 carboxylate of the fatty acid (42), to demonstrate that the C-1 carboxylate of SCP-2-bound oleic acid was located in a solvent-accessible region, presumably near the surface of the protein. Unfortunately, this did not provide information regarding either the interaction of the other oleic acid carbons (adjacent versus remote to the C-1 carboxylate) or the degree of aqueous exposure of these carbons in unbound versus SCP-2-bound form. To more fully characterize the SCP-2 fatty acid binding site, the interaction of fully enriched [13C]oleic acid (designated [13C18]oleic acid) was determined. The 13C NMR spectrum (not shown) of [13C18]oleic acid was characterized by a single broad, intense resonance that could be attributed to the majority of the internal methylene carbons and a series of well resolved, J-coupled multiplets. The 13C NMR spectrum of oleic acid has been previously assigned (59); however, the important carbons for the following analysis are C-1 (δ, δ 179.4 ppm), C-2 (d, δ 35.3 ppm), and C-17 (t, δ 22.5 ppm).

The pH dependence of [13C18]oleic acid was determined in free and SCP-2-bound form. Over the pH range of 8.4–5.0, carbons C-1, C-2, and C-3 of free oleic acid all experience pH-dependent upfield chemical shifts, consistent with protonation of the terminal carboxylate (data not shown). In contrast, C-17 (as well as the remaining carbons) varied less than 0.1 ppm over the pH range of 5.0–8.4.

As in the case of free oleic acid above, [13C18]oleic acid bound to SCP-2 also displayed pH-dependent chemical shift behavior. Carbons C-1, C-2, and C-3 experienced upfield shifts with decreasing pH over the range of pH 7.7–5.8 (data not shown), consistent with titration of the C-1 carboxylate with an apparent $pK_a$ of 5.7. The direction, range, and, importantly, uniformity of all three curves (and the absence of any variability in the chemical shifts of the remaining carbons) further reinforce the
by the lack of significant change in the peak at δ 40.8 ppm. The addition of higher oleic acid levels to this sample was not possible due to solubility problems. These data suggest SCP-2 had two ligand binding sites. However, since SCP-2 bound cholesterol with much higher \( K_d \) near 4 nM (see above) than for LCFA (200–400 nM), the LCFA solubility problems prevented resolution of whether the cholesterol binding site also bound LCFA.

Displacement of SCP-2-bound [4-13C]Cholesterol by Oleoyl-CoA—To determine whether oleoyl-CoA bound at the same site(s) as cholesterol and LCFA, displacement of SCP-2-bound [13C]cholesterol and SCP-2-bound [13C]oleate by oleoyl-CoA was attempted. SCP-2 binds LCFA-CoA with \( K_d \) values of 2–4 nM (44), representing a 100-fold higher affinity for LCFA-CoA than LCFA and similar affinity to that of sterol (4 nM; see above). Therefore, the [4,13C]cholesterol/[13C\textsubscript{18}]oleic acid-SCP-2 complex in Fig. 2B was treated with 5 mM oleoyl-CoA. The subsequent 13C NMR spectrum (Fig. 2C) revealed complete abolition of the SCP-2-bound [13C\textsubscript{18}]oleic acid near 182 ppm, with a concomitant increase in unbound [13C\textsubscript{18}]oleic acid, as indicated by the upfield shift to 178 ppm and broadening of the C-1 and C-2 carbons of the labeled [13C\textsubscript{18}]oleic acid. In contrast, little or no reduction of the SCP-2-bound [4,13C]cholesterol at δ 40.8 ppm was observed in the presence of 5 mM oleoyl-CoA. Due to solubility problems, it was not possible to increase the oleoyl-CoA concentration further. These data were consistent with the presence of two SCP-2 ligand binding sites and with SCP-2 binding cholesterol with affinity near or higher than that of LCFA-CoA. However, the LCFA-CoA solubility problems precluded resolution of whether the cholesterol binding site also bound LCFA-CoA.

Influence of SCP-2 Concentration on Fatty Acid-Cholesterol Competitive Displacement—The results of the previous experiments were highly suggestive that SCP-2 has two separate binding sites, one with a higher affinity for fatty acid and a second with a higher affinity for cholesterol. To further confirm this possibility, a second attempt was made to displace SCP-bound cholesterol with oleic acid or oleoyl-CoA, but at lower SCP-2 concentration to further increase the concentration difference between the bound and displacing ligands. Accordingly, a 1:1 complex of SCP-2-[4,13C]cholesterol was prepared as before, and the sample was diluted to 200 μM, the lowest protein concentration that could be used without sacrificing substantial signal-to-noise in the NMR spectra. The sample was divided, and in separate experiments either [1,13C]oleic acid or oleoyl-CoA was added. As seen in Fig. 3, treatment of the 200 μM SCP-2-[4,13C]cholesterol complex (1:1) with 2 mM [1,13C]oleic acid, a 10-fold excess over bound [4,13C]cholesterol, again resulted in little or no displacement of bound cholesterol (Fig. 3B), although a new peak at 41.7 ppm, slightly downfield of the C-4 signal of [4,13C]cholesterol was observed. In contrast, the addition of 5 mM oleoyl-CoA to the 200 μM SCP-2-[4,13C]cholesterol complex resulted in nearly complete loss of the bound C-4 signal (Fig. 3C). Curiously, the new peak observed in the presence of 2 mM [1,13C]oleic acid, Fig. 3B, was also present in Fig. 3C, although in the case of oleoyl-CoA at a much higher intensity, rivaling that of the original intensity of C-4 of [4,13C]cholesterol observed in the aqueous cycloextrin inclusion complex (Table 1), leading to the speculation that this new peak may be a result of displaced cholesterol, if not free in solution then loosely associated on the surface of SCP-2.

Displacement of SCP-2-bound [1,13C]Oleic Acid by Cholesterol—To confirm the above displacement studies, the
reverse experiment was performed to determine whether cholesterol could displace previously bound fatty acid. Once again, [1-13C]oleic acid was the LCFA of choice, particularly since it was previously shown that at NMR concentrations (>1 mM) SCP-2 can bind 2 eq of oleic acid (42). Accordingly, a 2:1 complex of [1-13C]oleic acid bound to SCP-2 was prepared, and its NMR spectrum was determined (Fig. 4A). The sample was then transferred to an NMR tube containing 2 eq of solid [4-13C]cholesterol. After a short incubation at 37 °C, the resulting 13C NMR spectrum (Fig. 4B) indicated binding of cholesterol as denoted by the arrow in the upfield portion of Fig. 4B. Concomitantly, a 25% reduction in carboxylate intensity from SCP-2-bound [1-13C]oleic acid was observed in the downfield portion of the spectrum. Since the intensity observed for the bound [4-13C]cholesterol signal was lower than previously observed, an additional 2 eq of labeled [4-13C]cholesterol were added, and the NMR spectrum was reacquired (Fig. 4C). As shown in the upfield portion of Fig. 3C, the intensity of the SCP-2-bound [4-13C]cholesterol now more closely resembled that in previous experiments, while the bound oleate signal (downfield portion; Fig. 4C) was approximately 50% of its original intensity observed in Fig. 4A in the absence of cholesterol. Subsequent attempts to add additional cholesterol to the system led to no further changes in either 13C NMR signal intensity (data not shown). The results clearly indicate that in the millimolar oleic acid concentration range, SCP-2 did indeed bind 2 eq of oleic acid. Furthermore, upon being exposed to excess cholesterol, 1 eq of bound oleic acid was displaced from one site in favor of cholesterol.

Effect of pH on SCP-2-bound [4-13C]Cholesterol—While [1-13C]oleic acid bound to SCP-2 exhibited a pH dependence consistent with dissociation of the [1-13C]oleic acid from SCP-2 at acidic pH (see above), it is not known if the neutral cholesterol molecule would experience similar pH sensitivity while bound to SCP-2. All of the previous [4-13C]cholesterol complexes observed in this study were prepared at pH values greater than 7.0 in order to maximize competitive binding of oleic acid as shown for the SCP-2-[1-13C]oleic acid-[4-13C]cholesterol complex reported in Fig. 5A. Upon lowering the pH of this ternary complex to 6.3, the expected dissociation of [1-13C]oleic acid from the SCP-2-[1-13C]oleic acid-[4-13C]cholesterol complex was observed (Fig. 5B). In contrast, there was no loss in intensity of the C-4 signal of SCP-2-bound [4-13C]cholesterol. Even at a pH near 5.0, little appreciable change in intensity was observed for the SCP-2-bound [4-13C]cholesterol signal. Moreover, no change in the chemical shift of the C-4 signal of the SCP-2-bound [4-13C]cholesterol was observed at lower pH. Taken together, these results suggest minimal pH-induced changes in the SCP-2 cholesterol binding site adjacent to the hydroxyl end of the cholesterol molecule.

Displacement of Bound [1-13C]Oleic Acid by Oleoyl-CoA—The previous experiments demonstrated the ability of chole-
terol to displace [1-13C]oleic acid from one of the two SCP-2 ligand binding sites. Furthermore, oleoyl-CoA was shown in separate experiments to displace both [1-13C]oleic acid and [4-13C]cholesterol from the binding site. However, this did not directly demonstrate that oleic acid and oleoyl-CoA competed for the same two sites. Accordingly, a 2:1 complex of [1-13C]oleate and SCP-2 was prepared (Fig. 6A). The addition of a slight excess of oleoyl-CoA clearly demonstrated the complete displacement of both equivalents of labeled oleate, even at near equivalent ligand concentrations (Fig. 6B).

Size Distribution of Unliganded and Liganded SCP-2 Determined by Photon Correlation Spectroscopy—It must be considered that the existence of two, rather than one ligand binding site, determined by the above 13C NMR experiments was due to the high SCP-2 concentrations (0.3–1.5 mM) required for 13C NMR. To resolve this issue, the size distribution of the 1 mM SCP-2, 2 mM oleoyl-CoA, and 1 mM SCP-2 plus 2 mM oleoyl-CoA was determined by photon correlation spectroscopy as described under “Experimental Procedures” (Fig. 7). The mean peak size of unliganded 1 mM SCP-2 (Fig. 7A) was 36.0 ± 1.2 Å (mean ± S.D.). The distribution in size ranged from about 2.5 to 6 nm, consistent with the ellipsoidal shape of monomeric SCP-2 reported using fluorescence techniques at very low (nanomolar to micromolar) SCP-2 concentrations (44, 61). The mean peak size of 2 mM oleoyl-CoA micelles, 83.0 ± 3.4 Å (mean ± S.D.) (Fig. 7B) was 2.3-fold larger than that of SCP-2. Furthermore, the larger oleoyl-CoA micelles ranged up to 120 Å. In contrast, the mean peak size of 1 mM SCP-2 in the presence of 2 mM oleoyl-CoA, 40.0 ± 1.0 Å (mean ± S.D.) (Fig. 7C) was nearly the same as that of unliganded 1 mM SCP-2. This was consistent with most of the oleoyl CoA being bound to SCP-2 rather than in micellar form with SCP-2 adsorbed to the surface of the micelle. In summary, the light scatter data were consistent with neither unliganded SCP-2 nor liganded SCP-2.
being in aggregated or multimeric form at the millimolar concentrations necessary for \(^{13}C\) NMR experiments detailed above.

**DISCUSSION**

The molecular interrelationships of SCP-2's ability to bind LCFA and LCFA-CoA versus its interaction with cholesterol have not been examined. The primary objective of the present investigation was to examine these ligand binding site(s) in SCP-2, determine whether the protein had a single or multiple ligand binding sites, and explore the interrelationship(s) of the LCFA, LCFA-CoA, and cholesterol binding site(s). Several new findings contribute to our understanding of the molecular mechanism(s) whereby SCP-2 can function.

First, both the \(^{13}C\) NMR and fluorescent sterol data demonstrated for the first time that SCP-2 binds sterols with saturation binding and high affinity at a single binding site. NMR techniques were particularly valuable in that binding of sterol could be monitored without separation of bound from unbound sterol. In addition, the \(^{13}C\) nucleus represents a nonperturbing probe. SCP-2 efficiently solubilized [4-\(^{13}C\)]cholesterol directly from a suspension of the sterol, resulting in the observation of a new peak at 40.8 ppm. The results presented with [4,\(^{13}C\)]cholesterol demonstrating a single cholesterol binding site in SCP-2 were confirmed with a fluorescent sterol binding assay. NBD-cholesterol clearly demonstrated that SCP-2 bound cholesterol at a single cholesterol binding site with high affinity, \(K_d = 4.1 \text{nM}\). These studies represent a significant advance over our previous investigation (31), wherein a lipidex 1000 radiolabeled cholesterol competition assay and a fluorescent dehydroyrosterol assay were used. Because saturating binding of sterol to SCP-2 was not obtained with either a [\(^{13}C\)]cholesterol/lipidex 1000 radioligand binding assay or a dehydroergosterol fluorescence binding assay, accurate determination of stoichiometry of binding and \(K_d\) was not possible.

Second, the displacement studies demonstrated that the SCP-2 sterol binding site also bound LCFA-CoA and LCFA. Taken together with our previous data on affinities of SCP-2 for LCFA and LCFA-CoA, the relative order of ligand affinities at this site was suggested to be cholesterol > oleoyl-CoA > oleic acid. It is of interest to note that the other cellular protein thought to be involved in intracellular cholesterol trafficking, caveolin, also binds LCFA (62), and its oligomerization in vitro is stabilized by LCFA-CoA or cholesterol (63). These data suggest that intracellular cholesterol and fatty acid trafficking or metabolism may potentially be interrelated or regulated at least in part by proteins such as SCP-2 and/or caveolin.

Third, the NMR studies revealed that SCP-2 also had a second ligand binding site. This site bound LCFA and LCFA-CoA but not cholesterol. In contrast, earlier studies with SCP-2 binding of fluorescent LCFA (41–43) and fluorescent LCFA-CoA (44) revealed only a single ligand binding site. These data are not readily explained by nonspecific binding, since the second site did not bind cholesterol. The following two alternate explanations may be considered. (i) An additional binding site might be formed by SCP-2 aggregation/oligomerization at the high SCP-2 concentration used for NMR studies (millimolar) versus fluorescence studies (<1 \(\mu\)M). However, the light scattering data presented herein demonstrated that this was not the case. At 1 \(\text{mM}\) SCP-2, without and with oleoyl-CoA, SCP-2 had mean peak sizes of 36 and 40 Å, respectively (Fig. 7). Similarly, fluorescence studies of <1 \(\mu\)M SCP-2 showed that unliganded SCP-2 and SCP-2 with oleoyl-CoA had hydrated diameters of 41 and 45 Å, respectively (44). These diameters, determined by either light scattering at 1 mm protein or fluorescence at <1 \(\mu\)M protein were all essentially the same as the hydrodynamic diameter theoretically predicted for a monomeric 13.2-kDa protein, i.e. SCP-2 (44). The range in light scatter distribution of 1 mM SCP-2, from 25–58 Å, was also consistent with the fact that SCP-2 is not perfectly spherical but is somewhat ellipsoidal as previously determined by fluorescence techniques (44, 61). Thus, the light scatter data demonstrated that both unliganded SCP-2 and liganded SCP-2 were not aggregated or in multimeric form at the millimolar concentrations necessary for \(^{13}C\) NMR experiments. Therefore, it is unlikely that the second ligand binding site (LCFA, LCFA-CoA binding site) was due to aggregated/multimeric SCP-2. (ii) The second ligand binding site, which binds only LCFA and LCFA-CoA, may not be detectable by fluorescence if it is located in SCP-2 in a site that has much lower hydrophobicity than the first ligand binding site. There is considerable support for this possibility in studies with the cytosolic fatty acid proteins, where similar phenomena have been observed. For example, at micromolar concentration, the intestinal fatty acid-binding protein, I-FABP, bound only fluorescent fatty acid and not fluorescent fatty acyl-CoA in a 1:1 stoichiometry (i.e. a single ligand binding site exclusive for fatty acids) (36, 64). However, at micromolar I-FABP concentration, a second ligand binding site in I-FABP was revealed by displacement of bound fluorescent fatty acid with LCFA-CoA and by binding experiments with micromolar concentrations of ADIFAB, a covalently “tagged” I-FABP. Similarly, NMR and x-ray, but not fluorescence, studies of the liver fatty acid-binding protein, L-FABP, revealed additional ligand binding sites. At micromolar concentrations, the L-FABP bound 2 mol of fluorescent LCFA/mol of protein (58, 65, 66), while at micromolar NMR (67) and x-ray crystal (68) concentrations, the L-FABP had more than two ligand binding sites. Finally, there are as yet very little data regarding the location of either ligand binding site in SCP-2.

Fluorescence energy transfer experiments between the SCP-2 tryptophan residue and bound fluorescent fatty acid indicated that the fatty acid fluorophore was located at an average distance of 40 Å from the SCP-2 tryptophan (43). However, it is as yet not possible to correlate which of the two fatty acid binding sites determined by NMR was actually detected by the fluorescence energy transfer experiment.

If the second SCP-2 ligand binding site were a concentration phenomenon, rather than being due to a difference in methodology, the significance of the SCP-2 ligand binding site is not clear. This would especially be the case if SCP-2 were evenly distributed in the cytosol of cells, under which condition it would represent about 0.01–0.1% of cytosolic protein, i.e. about 4–40 \(\mu\)M, depending on cell type (reviewed in Refs. 2 and 69). However, immunocytochemistry has shown that SCP-2 is highly concentrated in peroxisomes, less so in mitochondria and endoplasmic reticulum, and lowest in cytoplasm (reviewed in Ref. 69). Thus, SCP-2 concentration in peroxisomes is up to 100-fold higher than in extraperoxisomal areas, thereby bringing SCP-2 concentrations into the millimolar range in this organelle. Furthermore, peroxisomes are involved in both \(\alpha\)-oxidation and \(\beta\)-oxidation of LCFA as well as cholesterol biosynthesis (reviewed in Refs. 70 and 71). Thus, both SCP-2 ligand binding sites may be physiologically important in the peroxisomes, especially if the appearance of the second ligand binding site is concentration-dependent, while in the other cellular sites (where SCP-2 is found at much lower concentrations) the SCP-2 cholesterol/LCFA/LCFA-CoA binding site would be expected to be more significant.

The finding that SCP-2 may have two ligand binding sites that differ significantly in their ligand specificity suggests that SCP-2 may be involved not only in cholesterol, but also in fatty acid, intracellular trafficking and metabolic targeting. Consistent with this possibility, results obtained from in vitro studies
gene-ablated animals (27, 29, 45, 75) clearly show that SCP-2 can modulate both cholesteryl ester and triacylglycerol formation. Since the LCFA-CoA and cholesterol may competitively inhibit one another's binding to SCP-2, this suggests the possibility that the type of SCP-2-ligand complex presented to microsomal enzymes may influence the metabolic targeting of the respective ligands: e.g. SCP-2-LCFA-CoA (used for both cholesteryl ester and glyceride synthesis) versus SCP-2-cholesterol (used for cholesteryl ester synthesis or cholesterol oxidation). Finally, both LCFA and LCFA-CoA are important signaling molecules (reviewed in Ref. 76) and regulate nuclear transcription factors (77, 78). One may speculate, therefore, that the competitive binding equilibria between cholesterol, LCFA, and LCFA-CoA for the SCP-2 ligand binding sites may also influence both signaling and nuclear transcription in the cell.

In summary, the ligand binding studies presented herein significantly contribute to our understanding of the physiological function of SCP-2. The data establish for the first time that SCP-2 has two ligand binding sites. First, one site binds cholesterol, LCFA, and LCFA-CoA. Second, in addition to the first site, at high SCP-2 concentrations a second ligand binding site appears that also binds LCFA and LCFA-CoA. Putative functions for SCP-2 binding both fatty acid and cholesterol are in regulation of intracellular cholesteryl ester and glyceride synthesis, a possibility supported by studies both in vitro and in vivo. SCP-2 stimulates several microsomal enzymes utilizing LCFA-CoA and/or cholesterol in vitro: 13-fold enhancement of glycerol-3-phosphate acyltransferase and 7-fold stimulation of LCFA-CoA cholesteryl acyltransferase (34, 72–74). Likewise, in intact transfected L-cells, SCP-2 expression stimulated 1.6-fold the microsomal esterification of [14C]cholesterol or [14C]cholesterol and increased the mass of intracellular cholesteryl esters and triacylglycerols by 1.3- and 2.0-fold, respectively (27). Overexpression of SCP-x and SCP-2 in transfected L-cells also increased cholesteryl ester formation from [14C]cholesterol or [14C]cholesterol by 2.3- and 2.5-fold, respectively (29). Finally, and as expected from the transfected L-cell studies, SCP-2 gene-ablated mice showed a 2-fold decrease in liver cholesteryl ester and triacylglycerol mass (45). In conclusion, these studies suggest that SCP-2 may play a major role in both cholesteryl and glyceride metabolism. It may be suggested that differential SCP-2-mediated effects on cholesteryl ester versus glyceride synthesis may be influenced by the relative occupancy of the SCP-2 ligand binding sites by cholesterol, LCFA, and LCFA-CoA.

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