Lipid Specificity and Location of the Sterol Carrier Protein-2 Fatty Acid-Binding Site: A Fluorescence Displacement and Energy Transfer Study[1]

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ABSTRACT: Although it was recently recognized that sterol carrier protein-2 (SCP-2) interacts with fatty acids, little is known regarding the specificity of SCP-2 for long-chain fatty acids or branched-chain fatty-acid-like molecules. Likewise the location of the fatty-acid binding site within SCP-2 is unresolved. A fluorescent *cis*-parinaric acid displacement assay was used to show that SCP-2 optimally interacted with 14–22 carbon chain lipidic molecules: polyunsaturated fatty acids > monounsaturated, saturated > branched-chain isoprenoids > branched-chain phytol-derived fatty acids. In contrast, the other major fatty-acid binding protein in liver, fatty-acid binding protein (L-FABP), displayed a much narrower carbon chain preference in general: polyunsaturated fatty acids > branched-chain phytol-derived fatty acids > 14- and 16-carbon saturated > branched-chain isoprenoids. However, both SCP-2 and L-FABP displayed a very similar unsaturated fatty-acid specificity profile. The presence and location of the SCP-2 lipid binding site were investigated by fluorescence energy transfer. The distance between the SCP-2 Trp^{50} and bound *cis*-parinaric acid was determined to be 40 Å. Thus, the SCP-2 fatty-acid binding site appeared to be located on the opposite side of the SCP-2 Trp^{50}. These findings not only contribute to our understanding of the SCP-2 ligand binding site but also provide evidence suggesting a potential role for SCP-2 and/or L-FABP in metabolism of branched-chain fatty acids and isoprenoids.
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Eukaryotic cells contain a variety of lipid transfer proteins (1–3). While some of these, such as fatty-acid binding proteins (FABP) and fatty acyl CoA binding protein, appear localized primarily to the cell cytoplasm (2,3), others such as sterol carrier protein-2 not only are found in cytoplasm but also are highly enriched in peroxisomes (4–7). The origin and function of the cytoplasmic form of SCP-2 are unclear at this time. Furthermore, the ligand specificities of many of the lipid transfer proteins are quite broad. This range of specificity is best exemplified by SCP-2, also called the nonspecific lipid transfer protein. SCP-2 is a soluble 13.2 kDa basic protein found in all mammalian tissues examined (8–13). Studies *in vitro* indicate that SCP-2 transfers nearly all phospholipids tested (except cardiolipin) (14,15), glycosphingolipids and gangliosides (16), and sterols (1,8,17–26). Furthermore, SCP-2 stimulates a variety of terminal enzymatic reactions in cholesterol biosynthesis and cholesterol esterification *in vitro* (8,9,18). Although the physiological function(s) of SCP-2 may relate to its ability to transfer the above lipids, this has been established only for cholesterol in permanently transfected L-cells (27) and in transiently transfected Chinese hamster ovary cells (28).

The location of the SCP-2 ligand binding site has not been identified. While the structure of apo SCP-2 has been determined by nuclear magnetic resonance (NMR) spectroscopy, that of the holo-SCP-2 has not been reported (29). Resolution of an SCP-2 ligand binding site has been difficult owing to: (i) Poor aqueous solubility and/or propensity of phospholipids and sterols to form membranous or micellar structures at very low, often nanomolar concentrations; (ii) Inability to obtain saturation binding of a putative SCP-2 ligand binding site with sterols or phospholipids *in vitro* (10,14,15,18,30,31); (iii) The use of organic solvents as a vehicle for the lipid ligands. Organic solvents such as ethanol and propylene glycol may interfere with the binding of ligands to SCP-2 (32); (iv) Loss of putative ligand(s) during the SCP-2 purification procedure. It was reported that SCP-2 purified from tissues has no bound ligand (33,34).

Recently, it was shown that SCP-2 has a fatty-acid binding site (32,35). The present investigation explores the acyl specificity and location of the SCP-2 fatty-acid binding site by use of a fluorescent displacement assay and fluorescence energy transfer from the SCP-2 intrinsic aromatic amino acid residue (Trp^{50}) to bound *cis*-parinaric acid (9*Z*,11*E*,13*E*,15*Z*-octadecatetraenoic acid) serving as an energy transfer acceptor. This study takes advantage of the recent observation that *cis*-parinaric acid binds to SCP-2 with saturation kinetics (32,35) and

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Abbreviations: SCP-2, sterol carrier protein-2; L-FABP, liver fatty-acid binding protein; NMR, nuclear magnetic resonance; *cis*-parinaric acid, 9*Z*,11*E*,13*E*,15*Z*-octadecatetraenoic acid.

the presence of a single aromatic amino acid residue, Trp^{50}, in SCP-2 (8).

MATERIALS AND METHODS

Materials. Cis-parinaric acid was obtained from Molecular Probes (Eugene, OR). *Cis*-parinaroyl CoA was synthesized and purified as described earlier (36). Dimethylallyl pyrophosphate, farnesyl pyrophosphate, geranyl pyrophosphate, geranyl geranyl pyrophospate, CoA (CoASH), phytanic acid, acyl-CoA synthase, and ATP were from Sigma Chemical Co. (St. Louis, MO). Saturated and unsaturated fatty acids were obtained from Nu-Chek-Prep Inc. (Elysian, MN). Phytenic acid was kindly provided by Drs. K. Tomer and Cary Weinberger (National Institutes of Health, Research Triangle Park, NC). Pristanic acid was a generous gift from Dr. A. Moser (Johns Hopkins University, Baltimore, MD). L-Tryptophan was from Calbiochem (San Diego, CA). All other chemicals were reagent grade or better.

Methods. Recombinant human SCP-2 and native rat liver fatty-acid binding protein (L-FABP) were isolated and purified as described earlier (37,38). L-FABP was delipidated as described (39). Protein purity was >99% as determined by silver-stained SDS polyacrylamide gel electrophoresis. SCP-2 and L-FABP concentration were determined by Bradford assay (40) (Bio-Rad Laboratories, Richmond, CA). L-FABP concentration determined by the Bradford assay was corrected according to amino acid analysis (41). The Bradford assay overestimates the L-FABP concentration by 1.69-fold.

Absorption and steady-state fluorescence spectroscopy. Absorption spectra were measured at room temperature (~24°C) using a UV/VIS Lambda 2 Double-Beam Spectrophotometer (Perkin-Elmer Inc., Norwalk, CT). Corrected (unless otherwise noted) steady-state fluorescence spectra were measured with an ISS PC1 photon counting spectrofluorimeter (ISS Instruments, Champaign, IL) in a quartz fluorescence cuvette, optical path length 1 cm. Temperature was maintained at 25°C (±0.1°C) in a thermostated cell holder. The excitation and emission bandwidths were 4 and 8 nm, respectively.

Displacement of cis*-parinaric acid bound to SCP-2 or L-FABP by fatty acids*. SCP-2 (0.18 μM) or L-FABP (0.10 μM) in 25 mM phosphate buffer (pH 7.4) were incubated with 0.44 and 0.30 μM *cis*-parinaric acid, respectively, for 5 min at 25°C to obtain stable fluorescence. Protein-bound *cis*-parinaric acid was displaced by fatty acids (3.0–4.5 μM) with acyl chain lengths ranging from 10 to 20 carbons. After addition of displacing fatty acid, the sample was allowed to equilibrate for 5 min before measurement of *cis*-parinaric acid fluorescence.

The form in which the fatty acids were added to the incubation mixture was important owing to the relatively low solubility of long-chain fatty acids in aqueous buffer. The critical micellar concentration of long-chain fatty acids is in the micromolar range (42,43). Thus, displacement by a very soluble medium-chain fatty acid (C_{10}) would not be complicated by the presence of micellar equilibria while, in contrast, the displacement by the long-chain fatty acid (C_{20}) would be complicated by multiple equilibria reflecting the affinities of both the micelle and the SCP-2 for the fatty acid. To minimize these complications related to the differential solubility of the displacing fatty acids, all displacing fatty acids were used as their sodium salts. The critical micellar concentration for the C_{10}–C_{20} fatty acid salts is 1000-fold higher, ~ several millimolar (44), as compared to the corresponding free fatty acids. Fatty acid sodium salts were prepared by dissolving free fatty acids in 10 mM NaOH. It should be noted that fatty acids with chain lengths shorter than C_{10} did not displace bound ligands (data not shown). Measurements were corrected for the blank (ligand or protein only) and for photobleaching (<1%).

Fluorescence quantum yield. Fluorescence quantum yield of SCP-2 in phosphate buffer (ϕ_{prot}) was measured by a relative method (45) as:

$$\phi_{prot} = \phi_{ref}\left[\frac{1-10^{-D_{ref}}}{1-10^{-D_{prot}}}\right]\left(\frac{S_{prot}}{S_{ref}}\right)\left(\frac{n_{prot}}{n_{ref}}\right)^2 \quad [1]$$

where ϕ_{prot} and ϕ_{ref} are the protein and the reference fluorescence quantum yield, respectively; S_{prot} and S_{ref} are integral emission of the protein and the reference, respectively; D_{ref} and D_{prot} are optical densities at excitation wavelength for the reference and the protein, respectively; and n_{prot} and n_{ref} are the respective protein and reference refractive indices. L-Tryptophan in phosphate buffered saline was used as the reference, $\phi_{ref} = 0.14$ at 25° (46). The value of n_{prot} was assumed to be 1.4 (47). The relative error in the measurement of ϕ_{prot} was ≤0.02.

RESULTS

Fatty-acid specificity of the SCP-2 and L-FABP fatty-acid binding sites. Cis-parinaric acid is a naturally occurring 18-carbon, kinked-chain, fluorescent analog of oleic acid that fluoresces poorly in aqueous solution, possibly owing to strong electrostatic interactions of this fluorophore with water molecules. When bound to SCP-2 or L-FABP, *cis*-parinaric acid fluoresces strongly, and Scatchard analysis indicates this fatty acid binds to SCP-2 and L-FABP with 1:1 and 2:1 stoichiometry, respectively. The respective binding affinities were estimated to be $K_d = 0.18$ and 0.41 μM, respectively (32,35).

The *cis*-parinaric acid binding assay was adapted to examine fatty-acid specificity of SCP-2 binding site *via* displacement of bound *cis*-parinaric acid. As shown in Figure 1, SCP-2 bound *cis*-parinaric acid was displaced by a variety of 10–22 carbon length fatty acids. However, this displacement ability was highly dependent on the chain length and degree of saturation of the various fatty acids. Among the saturated fatty acids, the C_{14} and C_{16} fatty acids were the most efficient displacers while shorter- or longer-chain fatty acids were very poor displacers (Fig. 1A). The monounsaturated fatty acids of equal chain length had approximately similar ability as the corresponding saturated fatty acids to displace SCP-2 bound *cis*-parinaric acid (Fig. 1B vs. 1A). In contrast, the polyunsat-

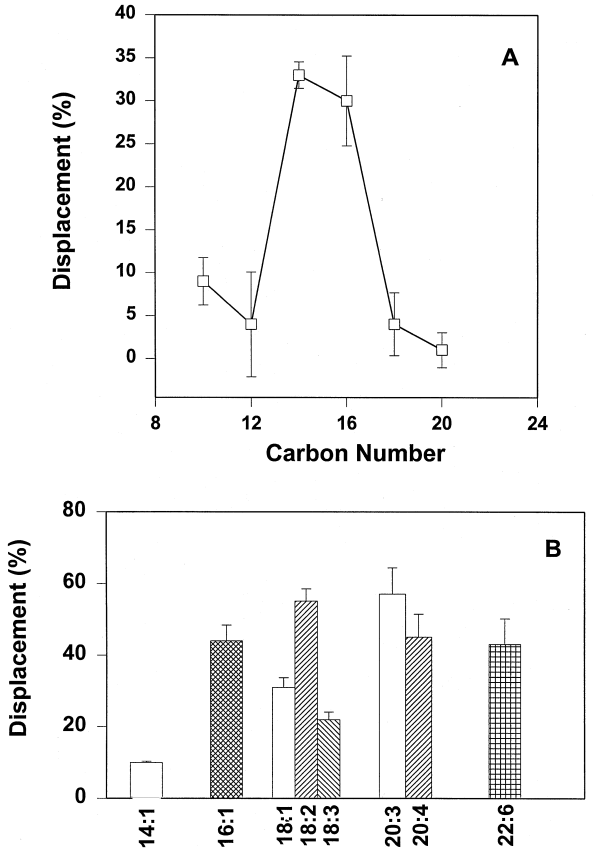

FIG. 1. Displacement of sterol carrier protein-2 (SCP-2) bound *cis*-parinaric acid by fatty acids. SCP-2 (0.18 μM) was preincubated with *cis*-parinaric acid (0.44 μM) followed by addition of displacing fatty acid (4.5 μM) as described in the Methods section. Values represent the mean ± SE (n = 3).

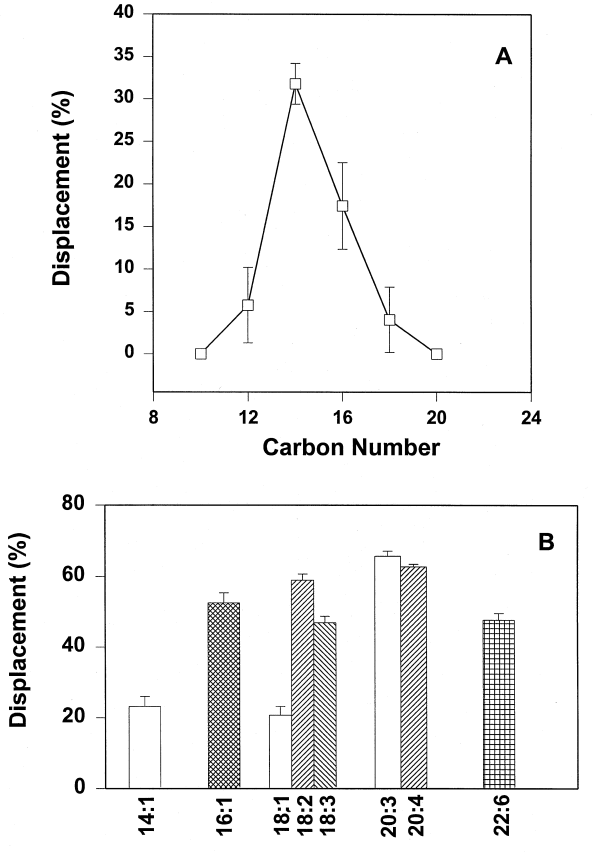

FIG. 2. Displacement of liver fatty-acid binding protein (L-FABP) bound *cis*-parinaric acid by fatty acids. L-FABP(0.1 μM) was preincubated with *cis*-parinaric acid (0.3 μM) followed by addition of displacing fatty acid (3 μM) as described in the Methods section. Values represent the mean ± SE (n = 3).

urated fatty acids were in general much more efficient displacers than the corresponding chain length saturated fatty acids (Fig. 1B vs. 1A).

The *cis*-parinaric acid binding assay was also applied to examine fatty-acid specificity of L-FABP *via* displacement of bound *cis*-parinaric acid. As shown in Figure 2, L-FABP bound *cis*-parinaric acid was readily displaced by a variety of 12–22 carbon length fatty acids. This displacement ability was also highly dependent on the chain length and degree of saturation of the various fatty acids. Among the saturated fatty acids, the C_{14} and C_{16} fatty acids were the most efficient displacers while shorter- or longer-chain fatty acids were very poor displacers (Fig. 2A). This pattern was very similar to that observed for SCP-2 (Fig. 1A). The monounsaturated fatty acids of equal chain length had approximately equal or similar ability as the corresponding saturated fatty acids to displace L-FABP bound *cis*-parinaric acid (Fig. 2B vs. 2A). In contrast and as observed for SCP-2, the polyunsaturated fatty acids were, in general, much more efficient displacers of L-FABP-bound *cis*-parinaric acid than the corresponding chain length saturated fatty acid (Fig. 1B vs. 1A).

Interaction of isoprenoids with the SCP-2 and L-FABP fatty-acid binding site. The *cis*-parinaric acid displacement assay was applied to further examine the specificity of SCP-2 and L-FABP fatty-acid binding sites for other hydrophobic molecules such as isoprenoids. Isoprenoids such as dimethylallyl-PP (C_5), farnesyl-PP (C_{10}), geranyl-PP (C_{15}), and geranyl geranyl-PP (C_{20}) are branched-chain aliphatic molecules that are intermediates in the microsomal and/or peroxisomal synthesis of cholesterol, dolichol, and ubiquinone. They are basically comprised of isopentyl units with increasing chain length. As shown in Table 1, the pyrophosphate derivatives of C_5 to C_{20} isoprenoids displaced SCP-2 and L-FABP bound *cis*-parinaric acid. Unlike the fatty acid displacers (Figs. 1 and 2), short-chain isoprenyl pyrophosphates efficiently displaced SCP-2 bound, and less so L-FABP bound, *cis*-parinaric acid (Table 1). In both cases the geranyl geranyl pyrophosphate was a more efficient displacer than the shorter-chain isoprenyl pyrophosphates. Surprisingly, isoprenyl pyrophosphates with chain length from 4–12 were much more efficient in displacing SCP-2 than L-FABP bound *cis*-parinaric acid (Table 1). However, the longest chain length C_{16} geranyl geranyl pyrophosphate was equally effective in displacing SCP-2 and L-FABP bound *cis*-parinaric acid (Table 1). These data suggest that both SCP-2 and L-FABP may play different role(s) in cellular metabolism of isoprenoids.

TABLE 1
Displacement of *cis*-Parinaric Acid Bound to Fatty Acid-Binding Proteins by Isoprenoids[a]

	Displacement (%)	
Displacing agent	SCP-2	L-FABP
Dimethylallyl pyrophosphate	34 ± 2.3	8 ± 0.9
Farnesyl pyrophosphate	40 ± 1.7	17 ± 2.1
Geranyl pyrophosphate	34 ± 2.1	13 ± 1.4
Geranyl geranyl pyrophosphate	65 ± 1.2	68 ± 0.9

[a]Sterol carrier protein-2 (SCP-2) and liver fatty-acid binding protein (L-FABP) (0.18 μM) were preincubated with *cis*-parinaric acid (0.45 μM) followed by the addition of displacing ligands (4.5 μM). Values represent mean ± SEM (n = 3–5).

Interaction of branched-chain fatty acids with the SCP-2 and L-FABP fatty acid-binding site. The *cis*-parinaric acid displacement assay was applied to examine if SCP-2 and L-FABP also interacted with branched-chain fatty acids such as those derived from phytol. In ruminants, the phytol side-chain of chlorophyll is cleaved to release phytol which is oxidized to phytanic acid by enteric bacteria such that both the phytanic acid and its precursor phytol are present in human diets containing ruminant fats and dairy products (48). Daily human consumption of phytanic acid is 50–100 mg (49). Phytanic acid is absorbed, transported to the liver, and metabolized (oxidized) in peroxisomes (50). Normal human serum levels of phytanic and phytenic acid are 6 and 2 μM, respectively (51). The C_{16} carbon phytol-derived fatty acids displaced SCP-2 bound, more so than L-FABP, bound *cis*-parinaric acid (Table 2). Phytenic acid displaced both SCP-2 and L-FABP bound *cis*-parinaric acid to the same degree, 22 and 26%, respectively (Table 2). In contrast, phytanic acid and pristanic acid were more effective in displacing L-FABP than SCP-2 bound *cis*-parinaric acid (Table 2). These data suggest that both SCP-2 and L-FABP may play different role(s) in branched-chain fatty-acid metabolism within the cell.

Location of SCP-2 fatty-acid binding site as determined by fluorescence energy transfer from SCP-2 to the bound cis*-parinaric acid*. Although the structure of the L-FABP fatty-acid binding sites was recently reported by X-ray crystallography (52), similar progress has not yet been made with regard to the SCP-2 fatty-acid binding site. The nucleotide sequence of SCP-2 indicates the presence of a single Trp at position 50 and no tyrosine residues in the SCP-2 polypeptide chain (28). Hence, the Trp^{50} may serve as an internal "ruler" or reference point for locating the putative fatty-acid binding site in SCP-2. The proximity of SCP-2 Trp^{50} to the fatty-acid binding site can be determined by fluorescence energy transfer from Trp^{50} to a bound *cis*-parinaric acid. The absorbance spectrum of the SCP-2 bound *cis*-parinaric acid was determined at a fatty acid to protein molar ratio of 1:10, which corresponds to approximately 90% of the fatty acid bound to SCP-2. The unbound free *cis*-parinaric acid poorly absorbs in aqueous buffers owing to strong electrostatic interactions with water molecules. These factors allowed determination of the absorption spectrum of SCP-2 bound *cis*-parinaric acid by measuring the differential spectrum with SCP-2/*cis*-parinaric acid in the sample and SCP-2 in the reference. This explains why the protein absorption component is not shown in Figure 3. The absorbance spectrum of *cis*-parinaric acid bound SCP-2 displayed maxima near 292, 306, and 320 nm (Fig. 3, dashed line). The nonzero absorbance of *cis*-parinaric acid at 250 nm was due to nonactive absorbance of the aqueous buffer which was very difficult to compensate owing to strong absorption of water in that spectral region.

Upon excitation at 310 nm, a nonstructured emission spectrum was observed with a peak at ~416 nm (data not shown). As shown in Figure 3 (solid line), the SCP-2 Trp^{50} fluorescence emission spectrum significantly overlaps the absorption spectrum of *cis*-parinaric acid. This provides the resonance conditions required for remote dipole-dipole electron excitation energy transfer (Forster energy transfer) from the SCP-2 Trp^{50} (donor) to the bound *cis*-parinaric acid (acceptor). Forster energy transfer from Trp to *cis*-parinaric acid has been previously used to measure interatomic distances in intestinal FABP (53). The efficiency of Forster energy transfer from SCP-2 Trp^{50} to *cis*-parinaric acid was examined in experiments where SCP-2 was excited at 255 nm, and SCP-2 Trp^{50} fluorescence emission at 332 nm was monitored upon titration of SCP-2 with increasing amounts of *cis*-parinaric

TABLE 2
Displacement of *cis*-Parinaric Acid Bound to Fatty Acid-Binding Proteins by Phytol Derivatives[a]

	Displacement (%)	
Displacing agent	SCP-2	L-FABP
Phytenic acid	22 ± 0.6	26 ± 0.7
Phytanic acid	28 ± 2.6	48 ± 1.0
Pristanic acid	26 ± 2.4	48 ± 0.9

[a]SCP-2 and L-FABP (0.18 μM) were preincubated with *cis*-parinaric acid (0.45 μM) followed by the addition of displacing ligands (4.5 μM). Values represent mean ± SEM (n = 3–5). See Table 1 for abbreviations.

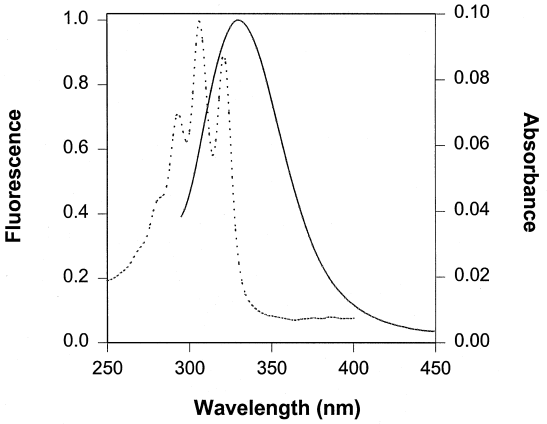

FIG. 3. Spectral characteristics of SCP-2 and *cis*-parinaric acid. Fluorescence emission spectra of SCP-2 (1 μM) in phosphate buffer, pH 7.4 (excitation at 280 nm, solid line). Absorption spectrum of *cis*-parinaric acid (1.3 μM) bound to SCP-2 (dotted line). See Figure 1 for abbreviation.

acid acceptor (Fig. 4A). SCP-2 Trp^{50} was excited at 255 nm because at this wavelength the acceptor, *cis*-parinaric acid, had negligible absorbance over the concentration range used in the titration experiments. As shown, upon titration of SCP-2 with increasing amounts of *cis*-parinaric acid, the tryptophanyl emission of SCP-2 gradually declined to ~40% of its initial level (legend to Fig. 4A). Such gradual decrease of tryptophanyl emission was accompanied by the appearance of an additional fluorescence emission band near ~420 nm (Fig. 4B) which can be attributed to the sensitized emission of *cis*-parinaric acid. It should be noted that no detectable SCP-2 fluorescence quenching was observed when the protein emission was monitored during SCP-2 titration with nonfluorescent oleic acid (data not shown). This observation strongly suggests that SCP-2 tryptophanyl fluorescence quenching detected in the presence of fluorescent *cis*-parinaric acid cannot be explained by direct interaction of two fluorophores, or by conformational changes of the protein induced by a bound fatty acid. Therefore, taken together, the above observations regarding the SCP-2 fluorescence quenching in the presence of *cis*-parinaric acid are consistent with the predominant mechanism of this quenching process being fluorescence energy transfer from SCP-2 Trp^{50} to the bound *cis*-parinaric acid. The efficiency of SCP-2 fluorescence quenching by *cis*-parinaric acid was estimated as 60% (Fig. 4A). Based on this energy transfer efficiency, the critical distance of energy transfer, R_o, for the SCP-2 Trp^{50} and *cis*-parinaric acid donor/acceptor pair was calculated as (54):

$$R_o = (9.765 \times 10^3)(K^2JQn^{-4})^{1/6} \quad [2]$$

where Q is the quantum yield of the donor in the absence of the acceptor; n, the refractive index; J, the overlap integral is defined as:

$$J = \int F_D(\lambda)\varepsilon_A(\lambda)\lambda^4 d\lambda \Big/ \int F_D(\lambda)d\lambda \quad [3]$$

where $F_D(\lambda)$is the normalized emission spectrum of the donor; $\varepsilon_A(\lambda)$ is the absorption spectrum of acceptor. Finally K^2 is the orientation factor ranging from 0 to 4. Energy transfer efficiency, E, is related to the distance between the donor and the acceptor r by:

$$E = R_o^6 \Big/ (R_o^6 + r^6) \quad [4]$$

The values of the parameters describing the Forster energy transfer in the SCP-2 Trp^{50}/*cis*-parinaric acid donor/acceptor pair are presented in Table 3. The calculated intermolecular distance between SCP-2 Trp^{50} and the *cis*-parinaric acid, based on the assumption that $K^2 = 2/3$ (i.e., for random orientation of the donor and the acceptor moments) was $r = 40$ Å (Table 3).

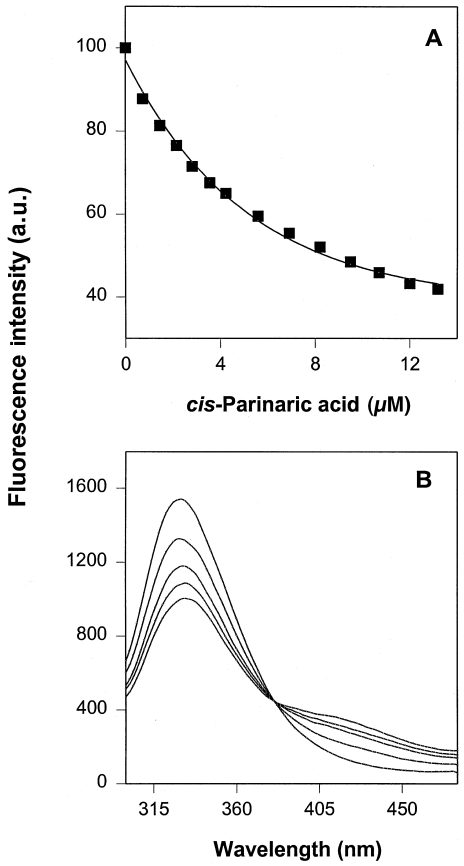

FIG. 4. Forster energy transfer between SCP-2 tryptophanyl (the donor) and bound *cis*-parinaric acid (the acceptor). (A) SCP-2 (1 μM) fluorescence quenching upon its titration with fatty acid. Excitation at 255 nm, emission at 332 nm. (B) Fluorescence emission spectra of SCP-2 (1 μM) in the presence of increasing amounts of *cis*-parinaric acid. From top to bottom: 1 μM SCP-2; 1 μM SCP + 0.5 *cis*-parinaric acid; 1 μM SCP + 1.0 μM *cis*-parinaric acid; 1 μM SCP + 1.5 μM *cis*-parinaric acid; 1 μM SCP + 2 μM *cis*-parinaric acid; 1 μM SCP + 2.5 μM *cis*-parinaric acid. Excitation at 255 nm. See Figure 1 for abbreviation.

TABLE 3
Forster Energy Transfer Parameters for Fluorescence Energy Transfer from SCP-2 Trp^{50} to Bound *cis*-Parinaric Acid (PA)[a]

Donor/acceptor	J (M^{-1} cm^3)	R_o[b] (Å)	E (%)	$r_{2/3}$[c] (Å)
Trp/*cis*-PA	9.82 10^{-15}	43	60	40

[a]J, overlap integral; R_o, critical distance at which 50% energy transfer occurs; E, energy transfer efficiency; $r_{2/3}$, actual distance between two residues, with the assumption $K^2 = 2/3$ (where K^2 is the orientation factor).
[b]Calculated for $\phi_{prot} = 0.27$; $n = 1.4$, and $K^2 = 2/3$, where ϕ_{prot} is quantum yield and n is refractive index.
[c]Calculated for $K^2 = 2/3$. See Table 1 for other abbreviation.

DISCUSSION

Liver hepatocytes and intestinal enterocytes both contain SCP-2 and L-FABP (reviewed in Ref. 55). The physiological function of neither protein is known. Therefore, a knowledge on the ligand binding specificities of these proteins is important to begin understanding their potential functional role(s). While SCP-2 is known to enhance the intermembrane transfer of cholesterol, glycolipids and phospholipids, identification of a ligand binding site has been problematic owing to the poor aqueous solubility of the ligands involved as well as their high affinity for membranes (8,9,18,56,57).

Only recently was it recognized that SCP-2 binds fatty acids (32,58). Specific binding of fatty acids was previously not detected with the standard Lipidex 1000 (Sigma Chemical Co.) radioligand competition binding assay, because of the sensitivity of the SCP-2 fatty-acid binding site to the organic solvent vehicle used in this competition assay (32,59). In contrast, the level of organic solvent used in the Lipidex 1000 assay did not interfere with fatty-acid binding to L-FABP (32,59). Furthermore, unlike the L-FABP whose X-ray crystal structure with bound fatty acid is now available (52), little is known regarding the specificity of SCP-2 for long-chain aliphatic molecules or the location of the SCP-2 fatty-acid binding site. Although previous studies have examined the fatty-acid specificity of L-FABP, the respective ligands were generally used as the free fatty acids rather than the fatty-acid salts (reviewed in Refs. 2,43,60–62). As pointed out previously (11) and in the introductory section, this may provide an apparent fatty-acid binding ability complicated by the solubility of the fatty acid.

The present investigation examined for the first time the specificity of the fatty-acid binding site of SCP-2 and L-FABP by use of sodium salts of the fatty acids. In a displacement assay involving protein-bound fluorescent *cis*-parinaric acid, a series of unbranched saturated and unsaturated fatty acids, isoprenoids, as well as branched-chain fatty acids were examined. The data demonstrated that SCP-2 bound a variety of fatty acids with a similar chain length and unsaturation specificity pattern as did L-FABP. Furthermore, both proteins bound a series of isoprenyl pyrophosphates and branched-chain fatty acids derived from phytol. These ligands were able to displace SCP-2 and L-FABP bound *cis*-parinaric acid to similar extents as did the normal fatty acids. However, the two proteins exhibited distinct differences in their binding of these branched aliphatic chain ligands. It should be noted that the displacement assay does not measure absolute binding affinities, but rather utilizes a fixed set of conditions to compare the relative ability of a series of lipophilic molecules to displace SCP-2 bound *cis*-parinaric acid. Conclusions regarding the absolute values of the respective ligand affinities cannot necessarily be made from displacement assays. This has been shown for fatty-acid vs. fatty acyl-CoA binding to SCP-2, wherein the binding affinity of SCP-2 for these ligands differs two orders of magnitude (35). However, when the displacement conditions were optimized for the lower-affinity ligand, fatty acid, the relative displacement of fluorescent fatty acid by both fatty acid and fatty acyl-CoA did not reflect the absolute differences in K_ds (35).

The significance of the data obtained with regard to SCP-2, which has been localized to peroxisomes (both interior and cytoplasmic surface) and to a lesser extent other organelles such as mitochondria and endoplasmic reticulum (5,6), is several-fold: First, SCP-2 may enhance import of fatty acids and/or fatty acyl-CoA into peroxisomes or mitochondria and thereby stimulate fatty-acid β-oxidation. Second, by binding phytol-derived fatty acids, SCP-2 may enhance import of branched-chain fatty acids into peroxisomes for α-oxidation. Third, there is growing evidence that peroxisomes contain most of the enzymes for cholesterol biosynthesis (63). Therefore, by binding isoprenyl pyrophosphates, SCP-2 may be involved in peroxisomal as well as microsomal cholesterol biosynthesis. Fourth, by binding a variety of short- and long-chain isprenyl pyrophosphates, SCP-2 may influence dolichol biosynthesis. Dolichol is an important molecule involved in glycosylation of proteins, and SCP-2 has already been shown to enhance the activity of microsomal *cis*-prenyltransferase (64). Fifth, experiments with transfected L-cell fibroblasts expressing SCP-2 have shown that the microsomal esterification of cholesterol is enhanced (65). Thus, by exhibiting fatty acyl chain selectivity, SCP-2 may be involved in the subsequent utilization of fatty acyl-CoA for microsomal esterification of fatty acids to cholesteryl esters. Similar considerations for L-FABP must be modified by its preferential location in cell cytosol and in the region of the endoplasmic reticulum (reviewed in Ref. 66). In summary, the interaction of SCP-2 as well as L-FABP with a wide variety of aliphatic molecules (branched-chain fatty acids, and isoprenoids) as well as normal fatty acids suggests new potential roles for these proteins in lipid metabolism.

Although SCP-2 stimulates the intermembrane transfer of many different lipids, including sterols, phospholipids, glycolipids, etc., it is unclear whether these activities actually require a ligand binding site in the protein. As a basic protein SCP-2 may, by binding to the anionic surface of membranes, disrupt membrane structure and thereby enhance the desorption of lipid molecules from the membrane. Recent findings, however, indicate that this is not likely for SCP-2 (23). In order to function in intermembrane sterol transfer, for example, SCP-2 must bind sterol (23). The location of this sterol binding site has not been determined. Neither is it known whether any of the other lipids transferred by SCP-2 also bind to the same or different sites in SCP-2.

The first major step in resolving a putative SCP-2 ligand binding site was the determination of the tertiary structure of the apo SCP-2. Based on NMR data, it was concluded that apo SCP-2 is comprised of a polypeptide chain with three α-helixes, a five-stranded β-sheet, with the rest as turn or random coil (29). SCP-2 Trp^{50} is located on one side of the SCP-2 molecule in the middle of β-sheet II. However, the orientation (facing the aqueous or the hydrophobic interior) of the tryptophanyl aromatic ring in β-sheet II is not known. On the opposite side of the molecule is located β-sheet V, representing the C-terminal β-sheet, to which is attached a randomly oriented "tail." The NMR data further show that the overall shape of apo SCP-2 is roughly spherical or slightly ellipsoidal (29). This is in agreement with recently presented data that the rotational correlation time and hydrodynamic radius of the entire SCP-2 protein are consistent with a nearly spherical or slightly elliptical protein (35). The apo SCP-2 Trp^{50} fluorescence dynamics showed an overall protein rotational correlation time of 8.4 ns and a hydrodynamic radius of 20 Å, which is very similar to that of the theoretical radius (18 Å) of a 13.2 kDa (the molecular weight of apo SCP-2)

globular protein. This was also confirmed by the measurements of the rotational dynamics of SCP-2 bound *cis*-parinaric acid, which yielded a hydrodynamic radius of 20.5 Å (35). These data indicate that SCP-2 is a globular, slightly elongated protein. In contrast, an early fluorescence study of apo SCP-2 Trp^{50} fluorescence dynamics measured a much longer rotational correlation time of 13.8 ns and suggested that apo SCP-2 is highly elliptical with an axial ratio of nearly 3:1 (31). The reason for the discrepancy between the latter study vs. the NMR and the present results is not clear. However, in the earlier work, the longer 13.8 ns rotational relaxation time was obtained at high SCP-2 concentration, 10 μM (31), and suggests the presence of SCP-2 dimers. Indeed, at concentrations of 10 μM (instead of the 0.18 μM used in the present work), we have observed a similar longer rotational relaxation time near 15 ns, indicative of SCP-2 dimers (data not shown).

The second major contribution to resolving the location of an SCP-2 ligand binding site came from results of site-directed mutagenesis (67). As mentioned above, the apo SCP-2 polypeptide chain is comprised of three α-helixes and a five-stranded β-sheet, one of which (β-sheet V) is on the opposite side of the SCP-2 Trp^{50} (located in β-sheet II) (29). Site-directed mutagenesis indicates that deletion and/or mutations in β-sheet V abolish SCP-2 transfer activity of sterols and phospholipids. Two other parts of the SCP-2 molecule (helix A and amino acids next to Cys^{71}) are very near the β-sheet V and are also necessary for sterol and phospholipid transfer activity (29). Although the site-directed mutagenesis data do not provide definitive proof, they suggest that a sterol and/or phospholipid binding site may be in a cleft formed by β-sheet V, a portion of helix A, and amino acids around Cys^{71}.

The present results show, for the first time, direct data on the location of the fatty-acid binding site in holo SCP-2. Earlier results from this laboratory showed that SCP-2 binds fluorescent fatty acids with saturation kinetics and high affinity, K_ds 0.2–0.4 μM (32,35). Nevertheless, the location of the SCP-2 fatty-acid binding site and/or its relation to putative cholesterol and/or phospholipid binding/transfer site(s) were not known. The data presented herein show that SCP-2 Trp^{50} is able to transfer energy to bound *cis*-parinaric acid with high efficiency near 60%. This tryptophan was not exposed to aqueous solvent, as shown by the emission maximum wavelength (λ = 332 nm), and by the lack of oleic acid effect on the Trp^{50} emission intensity/spectrum. Therefore, the aromatic ring of Trp^{50}, located on β-sheet II, appears to be turned inward to the hydrophobic core of the protein. Forster energy transfer showed that the estimated distance between SCP-2 Trp^{50} and bound *cis*-parinaric acid was near 40 Å, based on an assumed random orientation of the donor and the acceptor. The experimentally determined and theoretically calculated diameters for apo SCP-2 were 41 and 37.6 Å, respectively (35). The experimentally determined diameter for holo-SCP-2 containing bound oleic acid was 49 Å (35). It should be noted that the orientation of the fatty acid in the SCP-2 binding site is with the carboxylate exposed near the surface (35). An examination of the structure of *cis*-parinaric acid indicates that the tetraene fluorophore extends from the middle of the molecule to its methyl terminal of the *cis*-parinaric acid, i.e., about 8–10 Å away from the carboxyl terminus (35). This would suggest that the assumed orientation factor used to calculate the distance between SCP-2 Trp^{50} and bound *cis*-parinaric acid, 40 Å, was reasonable. More importantly, an intermolecular distance of 40 Å betweeen Trp^{50} and the fluorophore of *cis*-parinaric acid would place the fatty-acid binding site of SCP-2 on the opposite side of the SCP-2 from the β-sheet II Trp^{50}.

In summary, SCP-2 interacted with a wide variety of aliphatic ligands: C_{12} to C_{14} fatty acids, C_{16} branched-chain fatty acids, and C_4 to C_{16} isoprenyl pyrophosphates. The SCP-2 fatty-acid binding site was located approximately 40 Å from (and on the opposite side of) the location of SCP-2 Trp^{50}. The latter observation is consistent with site-directed mutagenesis studies (67), indicating that the β-sheet V, opposite from Trp^{50}, is essential for sterol and phospholipid transfer activity. One interpretation of these facts is that the ligand binding site that interacts with fatty acids, sterols, and phospholipids may be the same site. These ligand binding data may provide new insights into the physiological importance of SCP-2 in the cell.

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