The Sterol Carrier Protein-2 Fatty Acid Binding Site: An NMR, Circular Dichroic, and Fluorescence Spectroscopic Determination†

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ABSTRACT: The interaction and orientation of fatty acids with recombinant human sterol carrier protein-2 (SCP-2) were examined by nuclear magnetic resonance (NMR), circular dichroism (CD), and fluorescence techniques. ^{13}C-NMR spectroscopy of stearic acid and oleic acid as well as fluorescence spectroscopy of *cis*-parinaric acid demonstrated that SCP-2 bound naturally occuring fatty acids with near 1:1 stoichiometry. Several findings indicated that the fatty acid was oriented in the binding site with its methyl end buried in the protein interior and its carboxylate exposed at the surface: the chemical shift of bound [18-^{13}C]-stearate; dicarboxylic/monocarboxylic acid *cis*-parinaric acid displacement; complete ionization of the carboxylate group of SCP-2 bound [1-^{13}C]stearate at neutral pH; lack of electrostatic interactions between ^{13}C-fatty acids with SCP-2 cationic residues; pH titratability of the SCP-2 bound [1-^{13}C]stearate carboxylate group. SCP-2 did not undergo global structural changes upon ligand binding or pH decrease as indicated by the absence of significant changes in NMR and only small alterations in time resolved fluorescence parameters. However, SCP-2 did undergo secondary structural changes detected by CD in the pH range 5−6. While these changes in secondary structure did not alter the fatty acid:SCP-2 binding stoichiometry, the affinity for fatty acid was increased severalfold at lower pH. In summary, ^{13}C-NMR, CD, and fluorescence spectroscopy provided a detailed understanding of the interaction of fatty acids with SCP-2 and further showed for the first time the orientation of the fatty acid within the binding site. The pH-induced changes in SCP-2 secondary structure and ligand binding activity may be important to the mechanism whereby this protein interacts with membrane surfaces to enhance lipid binding/transfer.

The sterol carrier protein-2 (SCP-2),[1] also called nonspecific lipid transfer protein, is a ubiquitous protein found in nearly all mammlian tissues examined (Wirtz, 1991; Reinhart, 1990; Moncecchi et al., 1991a; Vahouny et al., 1987). Unlike other intracellular lipid binding proteins, e.g., the 20-member fatty acid binding protein (FABP)[1] family, SCP-2 appears to be encoded by a single gene (Seedorf et al., 1994; Ohba et al., 1994; Moncecchi et al., 1991b; Seedorf & Assmann, 1991; Seedorf et al., 1993; Myers-Payne et al., 1996). This gene has two initiation sites that code for a 15 kDa pro-SCP-2 and 58 kDa SCP-x. The 15 kDa pro-SCP-2 is rapidly post-translationally cleaved to form the mature 13.2 kDa SCP-2.

While the physiological function of SCP-2 remains to be clearly resolved, a growing consensus implicates a role in the metabolism of a variety of lipids. A growing body of data supports a major function for SCP-2 in cellular cholesterol transport [reviewed in Schroeder et al. (1996a)]. Use of antisense techniques shows a role for SCP-2 in cholesterol transport into rat bile (Puglielli et al., 1995). Most of these investigations are based on *in vitro* assays of ligand transfer or enzyme stimulation. For example, SCP-2 enhances the *in vitro* intermembrane transfer of sterols (Woodford et al., 1994, 1995; Van Amerongen et al., 1989; Chanderbhan et al., 1982; Gadella et al., 1991; Frolov et al., 1996b, 1996c; Hapala et al., 1994; Schroeder et al., 1990a, 1990b), phospholipids [reviewed in Nichols (1988); Wirtz (1991); Gadella and Wirtz (1994); Schroeder et al. (1991, 1996)], and glycolipids [reviewed in Zilversmit (1984)]. Furthermore, SCP-2 stimulates the *in vitro* enzymatic conversion of 7-dehydrocholesterol to cholesterol, cholesterol esterification, bile acid biosynthesis, and progesterone formation [reviewed in Pfeifer et al. (1993a); Vahouny et al. (1987a); Szyperski et al. (1993a)]. Relatively few studies have been performed on SCP-2 function in intact cells wherein the only known genetic modification is in SCP-2 expression. Transfected cells in culture show that SCP-2 is involved in intracellular cholesterol trafficking (Moncecchi et al., 1996; Puglielli et al., 1995), mitochondrial cholesterol oxidation/steroidogenesis (Yamamoto et al., 1991), and microsomal cholesterol esterification.[2] Additional roles in

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[1] Abbreviations used: SCP-2, sterol carrier protein-2; L-FABP, liver fatty acid binding protein; NMR, nuclear magnetic resonance; CD, circular dichroism; *cis*-parinaric acid, 9*Z*,11*E*,13*E*,15*Z*-octadecatetraenoic acid; hexadecanoic acid; hexadecanedioc acid; dimethyl POPOP, 1,4-bis(4-methyl-5-phenyl-2-oxazolloyl)benzene.

[2] Murphy, E. J., & Schroeder, F. Sterol carrier protein-2 mediated cholesterol esterification in transfected L-cell fibroblasts. *Biochim. Biophys. Acta,* in press, 1996.

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fatty acid oxidation and esterification are also suggested. For example, SCP-2 is enriched in peroxisomes while SCP-x is exclusively found in peroxisomes, organelles involved in fatty acid and branched chain fatty acid oxidation (Lo et al., 1994; Keller et al., 1989; Van der Krift et al., 1985; van Heusden et al., 1990). In addition, the levels of triglycerides and triglyceride synthesis are markedly altered in transfected L-cell fibroblasts expressing SCP-2.[2] The potential involvement of SCP-2 in fatty acid oxidation and esterification is further supported by the observation that SCP-2 binds fatty acids (Schroeder et al., 1995) and fatty acyl CoAs (Frolov et al., 1996a; Gossett et al., 1996) with high affinity as indicated by K_d values near 0.2−0.4 M and 3−4 nM, respectively.

Despite this multitude of studies suggesting functional roles of SCP-2 in lipid metabolism, the exact structural basis for SCP-2 function *in vivo* or in intact cells is not known. Because of the difficulty in identification/characterization of a ligand binding site, this is a controversial area. For example, native SCP-2 purified from mammalian tissues does not contain bound cholesterol (Crain & Zilversmit, 1980; Chanderbhan et al., 1982). In addition, some reports suggest the absence of high-affinity binding of radio-labeled cholesterol (Wirtz & Gadella, Jr., 1990) or fluorescent dehydroergosterol (Gadella & Wirtz, 1991). Consequently, it was proposed that SCP-2 stimulates lipid transfer by simply enhancing membrane−membrane interactions or collisions (Wirtz & Gadella, Jr., 1990). In contrast, other observations are consistent with the presence of a lipid binding site in SCP-2 (Gadella & Wirtz, 1991; Gadella et al., 1991; Gadella & Wirtz, 1994; Nichols, 1987, 1988; Colles et al., 1995; Schroeder et al., 1990; Sams et al., 1991)[3] and that this ligand binding site is required for lipid transfer activity (Seedorf et al., 1994; Woodford et al., 1995). Native rat liver SCP-2 (Sams et al., 1991; Schroeder et al., 1990) and recombinant rat liver SCP-2 (Colles et al., 1995) both bind radio-labeled cholesterol and the fluorescent sterol dehydroergosterol with affinity in the micromolar range. Native rat liver SCP-2 (Nichols, 1987, 1988) and bovine liver SCP-2 (Gadella & Wirtz, 1991, 1994; Gadella et al., 1991) both bind fluorescent-labeled phospholipids. While K_d values have been reported in some of these studies (Colles et al., 1995; Schroeder et al., 1990), in the majority of cases the extremely low critical micellar concentration of cholesterol and dehydroergosterol (20−30 nM) (Schroeder et al., 1990b) as well as phospholipids resulted in low occupancy of SCP-2 ligand binding sites and inability to obtain K_d values and binding stoichiometry for these interactions. In contrast, both native as well as fluorescent fatty acids and fluorescent fatty acyl CoAs have μM to near mM critical micellar concentrations, thereby allowing saturation of SCP-2 binding sites, permitting the accurate determination of K_d values and binding stoichiometry (Frovlov et al., 1996a; Schroeder et al., 1995).

The present investigation was undertaken to obtain an in-depth understanding of SCP-2 fatty acid binding, with an emphasis on fatty acid orientation and pH sensitivity. ^{13}C-fatty acid NMR experiments avoided potential drawbacks of some fluorescent-labeled fatty acids that may not reflect the behavior of native nonfluorescent fatty acids. Circular dichroism provided a reliable measure of SCP-2 seondary structure changes. Time-resolved lifetime and anisotropy, as well as steady state fluorescence, using the naturally fluorescent *cis*-parinaric acid, yielded additional insights on fatty acid binding stoichiometry at very low concentrations and on potential changes in tertiary structure upon ligand binding. These data presented new insights on the binding of common fatty acids such as stearate and oleate to SCP-2, on the structure of the SCP-2 fatty acid binding site, and on the potential significance of SCP-2 secondary structure changes at low pH.

MATERIALS AND METHODS

Materials. [1-^{13}C]stearic acid, [18-^{13}C]stearic acid, and [1-^{13}C]oleic acid (99% ^{13}C-enriched) were purchased from Cambridge Isotope Labs (Andover, MA) as was perdeuterated dithiothreitol (DTT-d_{10}). *cis*-Parinaric acid was obtained from Molecular Probes, Eugene, OR. Oleic acid, heptadecanoic acid, palmitic acid, hexadecanedioc acid (thapsic acid), *p*-terphenyl, and (hydroxymethyl)aminomethane (Tris) were obtained from Sigma Chemical Co., St. Louis, MO. All other chemicals were reagent grade or better.

Methods. Recombinant human SCP-2 was isolated and purified as described earlier (Matsuura et al., 1993). The protein concentration was determined by Bradford assay (Bradford, 1976). It should be noted that all the recombinant SCP-2 proteins from a variety of species reported thus far contain at least one and sometimes more than one mutation near the N-terminus. For example, deletion of 10 or more amino terminal amino acids or certain substitutions at position 20 completely inactivated the recombinant SCP-2 in sterol transfer assays (Seedorf et al., 1994). While it is possible that the properties of the recombinant SCP-2 proteins may differ from those found endogenously in mammalian sources, the small differences in amino terminus amino acid sequence in the recombinant SCP-2 generally have not resulted in large functional differences: (i) Measured sterol carrier activity of recombinant human SCP-2 and native rat liver SCP-2 were almost identical (Noland et al., 1980; Seedorf et al., 1994) even though these proteins differ in their amino terminus (Pfeifer et al., 1993b). (ii) While recombinant mouse SCP-2 and native rat SCP-2 differ in three amino acids (Ser, Ser, Ala) in the amino terminus, they did not differ in ability to stimulate microsomal conversion of 7-dehydroergosterol to cholesterol (Moncecchi et al., 1991b). (iii) Likewise, recombinant mouse SCP-2 and native rat liver SCP-2 did not differ in ability to stimulate microsomal cholesterol esterification (Moncecchi et al., 1991b). (iv) SCP-2s from rat and human did not differ in ability to stimulate microsomal conversion of 7-dehydroergosterol to cholesterol, even though they differ in amino terminal amino acid sequence (Seedorf et al., 1994). (v) Recombinant human pro-SCP-2, containing a 20 amino acid amino terminal leader sequence, did not differ from recombinant SCP-2 in ability to stimulate microsomal conversion of 7-dehydroergosterol to cholesterol (Seedorf et al., 1994). (vi) Deletion of the amino terminal five amino acids in recombinant human SCP-2 did not affect sterol transfer and phospholipid transfer activities of the recombinant SCP-2 (Seedorf et al., 1994).

Lipid Extraction and Fatty Acid Analysis. 1-Butanol (6 mL) was added to 2 mL of 10 mM phosphate buffer, 2.5 mM dithiothreitol (pH 6.8) containing 200 nmol of recombinant human SCP-2. The samples were vortexed, mixed

by inversion for 15 min, and the phases separated by centrifugation at 1500 rpm for 10 min at 4 °C on an IEC PR6000 refrigerated centrifuge. The upper phase was transferred to a clean glass test tube and washed with 2 mL of double-distilled water. The 1-butanol (upper phase) was removed and evaporated to dryness under a stream of N_2. The residue was washed three times with 2 mL of hexane. The hexane was evaporated to dryness under a stream of N_2. Two milliliters of toluene:MeOH (1:1 v/v) containing 2% sulfuric acid was added, and the samples were incubated on a shaker at 65 °C for 4 h. The fatty acid methyl esters were extracted with 2 mL of petroleum ether. This extraction was then repeated. The petroleum ether phases were combined, dried under N_2, and resuspended in 4 mL of hexane containing heptadecanoic acid (C-17:0) as an internal standard. A separate tube containing a known amount of palmitic acid (C-16:0) was methylated to determine methylation efficiency. Fatty acid analysis was conducted by isothermal capillary gas chromatography using an SP-2330 column (Supelco, Bellefonte, PA) with a column temperature of 190 °C using a Model 14A gas liquid chromatograph equipped with dual flame ionization detectors (Shimadzu Inc., Kyoto, Japan). Values are expressed as the mean ± standard deviation of three separate determinations.

Preparation of Fatty Acid:SCP-2 Complexes for NMR. Complexes of fatty acids and SCP-2 were prepared in similar fashion to that described for fatty acid binding proteins (FABP) (Cistola et al., 1988, 1989, 1990). For each fatty acid sample prepared, a stock solution of SCP-2 (<5 mg/mL) was subjected to several buffer exchanges (20 mM KP, 4 mM DTT-d_{10}, pH 7.0 in 20% D_2O) via a YM3 Ultrafiltration membrane (Amicon, Beverly, MA), with a final concentration step yielding a volume of 0.4−0.45 mL, resulting in an final SCP-2 concentration of 15−20 mg/mL. ^{13}C-NMR reference spectra of the unliganded SCP-2 were acquired using this solution prior to fatty acid:SCP-2 complex formation. For the subsequent preparation of fatty acid:SCP-2 complexes, aliquots of the ^{13}C-labeled fatty acid [20 mM in $CHCl_3$:MeOH (2:1)] were added to a clean NMR tube, the solvent was removed under a stream of argon, and the residue was resuspended with gentle heating (35−40 °C) in an equal volume of 0.02 N NaOH. To this, the previous NMR sample (i.e., the initial unliganded SCP-2 or previous fatty acid:SCP-2 complex) was added, the sample mixed by several inversions of the NMR tube, and the complex quickly returned to the spectrometer for data acquisition.

^{13}C-NMR Spectroscopy. Proton-decoupled ^{13}C-NMR spectra were recorded at 125.76 MHz on a Bruker ARX-500 spectrometer equipped with a 5 mm C/H probe. Low-power ($^1/_4$ W) proton decoupling was accomplished using the powergated WALTZ sequence available in the Bruker software. The probe temperature was maintained within 0.1 °C using the Bruker VT-2000 controller without sample spinning. Routine acquisition parameters employed included the following: 1.0 s relaxation delay; 5 μsec pulse width (∼60° flip angle); 0.25 s acquisition time over 16 K data points. The data was zero-filled to 32 K and 10−15 Hz exponential line broadening applied prior to Fourier transformation. The strong resonance resulting from the ϵ-Lys/β-Leu residues were utilized as an internal chemical shift reference (39.45 ppm) (Gurd & Keim, 1973).

Time-Resolved Fluorescence Spectroscopy. Time-resolved fluorescence measurements were performed on a Model GREG 250 Subnanosecond Multifrequency Cross-correlation and Modulation fluorometer with KOALA sample compartment (ISS Instr., Champaign, IL). The excitation path contained a Glann−Taylor polarizer. The light source was an Innova−Sabre argon ion laser (Coherent Laser Group, Palo Alto, CA). This laser featured automatic wavelength selection in a multiline UV mode with the single lines at λ = 275.4, 300.2, 302.4, and 305.5 nm and continuous power output of 340, 630, 800, and 460 mW, respectively. The laser output was kept stable over several hours in either "Power Track" or the "Light Regulation" mode. In order to avoid appearance of artifacts due to scattered emission and Raman scatter, the protein fluorescence was observed from samples placed in a quartz fluorescence cuvette, optical length path = 0.3 cm (Expotech Inc., Houston, TX). Protein aromatic amino acid fluorescence emission was selected with 341 BP15 interference filters (Omega Optical Inc., Brattleboro, VT), transmittance maximum at λ = 341 nm (bandwidth = 15 nm). The excitation intensity was optimized to achieve a high signal to noise ratio and to minimize protein photobleaching to less than 10%. To avoid inner filter artifacts, sample absorbance at the excitation wavelengths was ≤0.05. All data were obtained in 20 mM Tris-HCl buffer at 25 °C.

Fluorescence Lifetime Data Acquisition and Analysis. Fluorescence decay kinetics were measured with the excitation polarizer oriented at 35° to yield rotation-free results (Spencer & Weber, 1970). Fluorescence lifetime data were acquired and analyzed at 12−15 modulation frequencies (20−225 MHz). External lifetime standard for measurement of protein fluorescence decay was *p*-terphenyl in absolute ethanol (τ = 1.05 ns). Data were acquired until the limit of standard error was reached: 0.04° and 0.2°, for phase and modulation, respectively. ISS-187 Software (ISS Inc., Champaign, IL) was employed for data collection and analysis. Fluorescence was analyzed by a sum of exponentials as: $I(t) = \sum_i f_i \exp(-t/\tau_1)$, where τ_i is lifetime and f_i is fractional intensity. The minimized χ^2 parameter was used as a criterion for the goodness of fit to the applied model. Generally, a χ^2 value less than 3 was considered to be acceptable (Parasassi et al., 1984).

The Anisotropy Decay Measurement and Analysis. Time-resolved anisotropy data were obtained at 12−15 modulation frequencies for both $I_{\parallel\parallel}$ and $I_\perp$ components over the range of 20−225 MHz with Glan−Taylor and Glan−Thompson polarizers in the excitation and emission channels, respectively. Standard error limits were set to 0.04, 0.2, and 0.001° for the measurement of phase, modulation, and polarization, respectively. The fluorescence anisotropy decay was modeled by a sum of exponentials as $r(t) = r_0 \sum g_i \exp(-t/\theta_i)$, where r_0 is anisotropy of a fluorophore in the absence of rotational diffusion, θ_i is the rotational correlation time and g_i is fractional anisotropy. Verification of the goodness of fit was performed as described above using ISS-187 Software (ISS Inc., Champaign, IL).

The equivalent hydrodynamic radius of the recombinant human SCP-2 was calculated as:

$$R = (3kT\theta/4\pi\eta)^{1/3} \quad (1)$$

where η is the solvent viscosity. The R value can be estimated also from the hydrated protein volume:

$$R = [(^3/_4\pi)(M/N_0)(V_1 + \delta_1 V_1)]^{1/3} \quad (2)$$

where M is the molecular weight of human recombinant SCP-2 (13.2 kDa); N_0 is Avogadro's number; δ_1 is the fraction of hydration; V_1 is the specific partial volume of a protein. $\delta_1 = 0.4$ g H_2O/g protein; $V_2 = 0.73$ cm^{-3} g^{-1} for an average protein (Cantor & Schimmel, 1980), and $V_1 = 1$ cm^3 g^{-1} for water in eq 2.

Circular Dichroism Measurements. Circular dichroic spectra of recombinant human SCP-2 (2 μM) dissolved in 10 mM Tris-HCl buffer were recorded in a 1 mm circular cuvette with the resolution of 1 nm on a J-710 spectropolarimeter (Jasco Inc., Easton, MD) at room temperature. The bandwidth was 2 nm, the sensitivity was 10 mdeg, the scan rate was 50 nm/min, and the time constant was 1 s. Usually, 10 scans were accumulated and the averaged scan was used for the secondary structure analysis. The protein secondary structure analysis was performed according to a self-consistent method (SELCON) (Sreerama & Woody, 1993). The corresponding software was provided by Jasco.

cis-Parinaric Binding Assay. The binding affinity of recombinant human SCP-2 for *cis*-parinaric acid was determined as previously described (Schroeder et al., 1995) with the following modifications. A 2 mL sample of 0.18 μM SCP-2 in Tris-HCl buffer was titrated with small increments of fatty acid (1−2 μL) dissolved in dimethylformamide. The latter was used instead of ethanol because ethanol significantly interferes with the ligand binding to SCP-2 (Schroeder et al., 1995). *cis*-Parinaric acid was added from a 301 μM stock solution in dimethylformamide. All measurements were performed at 25 °C.

Displacement of SCP-2 Bound cis-Parinaric Acid by Mono- and Dicarboxilic C-16 Fatty Acids. Recombinant human SCP-2 (0.18 μM) in phosphate buffer (pH 7.4) was incubated with *cis*-parinaric acid (0.45 μM) for 5 min at 24 °C until maximal fluorescence was obtained. The *cis*-parinaric acid bound to SCP-2 was displaced by the monocarboxylic hexadecanoic acid or by the dicarboxylic hexadecandioic acid at increasing concentration up to 9.24 μM displacing ligand. All fluorescence determinations were corrected for blanks (protein or ligand only) as well as for low levels of photobleaching.

RESULTS

Endogenous Fatty Acid Bound to Recombinant Human SCP-2. Endogenous bound fatty acid may compete with added ^{13}C-labeled fatty acids or *cis*-parinaric acid, interfering with acquisition of accurate spectra and binding parameters. Therefore, the fatty acids were extracted from SCP-2, converted to methyl esters, and analyzed by capillary gas chromatography as described in Methods. Only 1.61 nmol of fatty acids were bound per 200 nmol of SCP-2. Since SCP-2 has a single fatty acid binding site (Schroeder et al., 1995), less than 1% of the available binding sites of the recombinant human SCP-2 were occupied by fatty acids. The fatty acids bound to SCP-2 were primarily saturated fatty acids with 14, 16, 18, and 20 carbons (Table 1). The major components were palmitic (29% of total) and stearic acid (58% of total). Only one unsaturated fatty acid (14:1) was detected in low amounts representing 2.7% of total bound fatty acids.

Binding of ^{13}C-Labeled Fatty Acids to SCP-2. In the absence of bound fatty acid, SCP-2 is freely soluble in the 1−2 mM concentration range, forming a clear solution at neutral pH, and displayed a ^{13}C natural abundance NMR spectrum typical of a small protein, showing many narrow and partially resolved resonances in the aliphatic and aromatic carbon regions (not shown). Of particular interest to this study, however, the carbonyl region (Figure 1A) was defined by a broad grouping of resonances between 170 and 178 ppm associated with the more than 100 backbone and side chain amide carbonyl groups. A set of lower intensity signals was observed downfield of the main carbonyl envelope between 180−181 ppm and were attributed to a smaller group of ionized side chain carboxylates of aspartic and glutamic acid residues.

As shown in Figure 1B, the addition of a $^1/_2$ molar equivalent of [1-^{13}C]stearate to SCP-2 at pH 7.05 resulted in the observation of a new signal at 182.73. Subsequent addition of a second half-equivalent of ^{13}C-stearate (1:1 overall ratio SCP-2:fatty acid) resulted in a 2-fold increase in intensity of this signal (Figure 1C) with an observed line width of 18 Hz. The ^{13}C chemical shift and line width of this new signal is consistent with that found for the fatty acid bound to liver fatty acid binding protein (L-FABP)[1]

Table 1: Endogenous Fatty Acids of Recombinant Human SCP-2[a]

fatty acid	(nmol fatty acid/200 nmol SCP-2)
14:0	0.1238 ± 0.083
14:1	0.0441 ± 0.034
16:0	0.4682 ± 0.214
18:0	0.9413 ± 0.410
20:0	0.0330 ± 0.012

[a] Values represent the mean ± standard deviation ($n = 3$).

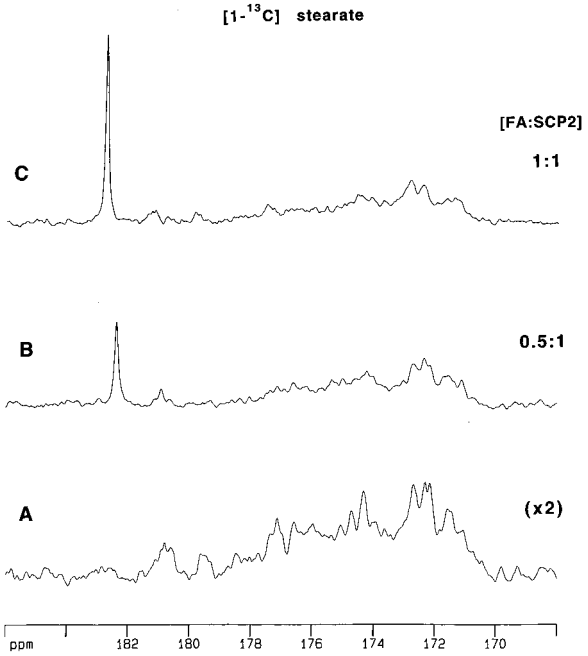

FIGURE 1: Downfield portion of the 125 MHz ^{13}C-NMR spectra of human SCP-2 detailing the carboxyl/carbonyl region in the absence (A) or presence (B,C) of bound [1-^{13}C]stearate. Spectrum A was recorded at 32 °C (pH 7.2) in the absence of fatty acid and is magnified 2-fold (relative to B and C) to highlight protein "background" signals originating from SCP-2 carbonyl carbons at natural abundance. Spectrum B was recorded after the addition of $^1/_2$ equiv of [1-^{13}C]stearate, and spectrum C was recorded after the adddition of a second $^1/_2$ equiv, resulting in a 1:1 complex between SCP-2 and stearate. Each spectrum was the result of 40−50, 000 accumulations.

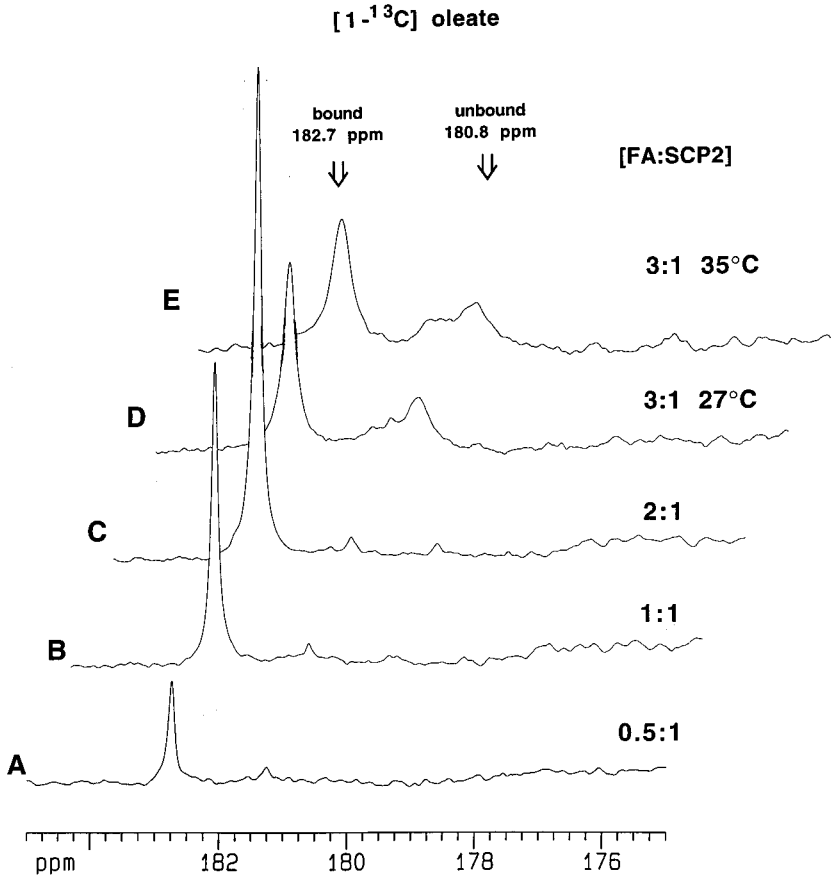

FIGURE 2: Carboxyl/carbonyl region of the ^{13}C-NMR spectra of [1-^{13}C]oleate:SCP-2 complexes at various fatty acid:SCP-2 mole ratios. Spectra A−D were recorded at 27 °C (pH 7.1−7.3) after the adddition of $^1/_2$, 1, 2, and 3 equiv of [1-^{13}C]oleate, respectively, resulting in the formation of 0.5:1 (A), 1:1 (B), 2:1 (C), and 3:1 (D) oleate:SCP-2 complex ratios. Spectrum E is of the 3:1 complex as in D, except it was recorded at 35 °C. The chemical shift of the bound oleate was invariant (182.72 ± 0.02 ppm) with concentration; however, each spectrum was offset to avoid overlap with adjacent spectra. The line width at half-height of each bound [1-^{13}C]oleate signal (182.7 ppm) is as follows: (A) 16, (B) 16, (C) 17, (D) 29 , and (E) 47 Hz. Each spectrum was the result of 40−50, 000 accumulations.

(Cistola et al., 1988). Additional aliquots of fatty acid led to no further changes observed in the ^{13}C-NMR spectra; however, a pronounced turbidity of the SCP-2 solution resulted, which quickly led to precipitation of crystalline fatty acid soap aggregates.

The binding of ^{13}C-labeled oleate to SCP-2 behaved initially in a similar fashion to that displayed by stearic acid. As shown in Figure 2 the addition of the first two half-equivalents of [1-^{13}C]oleate to SCP-2 resulted in a pronounced, rapid increase in the new signal at 182.74 ppm. Contrary to the case of stearate binding, however, the addition of a second full-equivalent of [1-^{13}C]oleate resulted in yet a further increase in signal intensity (Figure 2C), while the subsequent addition of a third equivalent lead to the observation of a new broad signal (∼50 Hz) at 180.8 ppm (Figure 2D) along with a concominant broadening of the original peak at 182.7 ppm. The observed chemical shifts of 182.7 and 180.8 ppm are in good agreement to that observed for L-FABP (Cistola et al., 1988), which were assigned respectively to bound and unbound forms of oleate. The latter assignment was confirmed in the absence of protein under identical conditions (data not shown). In addition, the increase in line width (17−29 Hz) of the bound resonance observed upon addition of the third equivalent of oleate is indicative of chemical exchange broadening due to interconversion between free and bound ligands. This was confirmed by raising the temperature of the fatty acid:SCP-2 complex to 35 °C, which resulted in additional broadening (Figure 2E) of both the bound and unbound signals, indicating the interconversion rate had increased from a slow to more intermediate rate of exchange on the NMR time scale.

To determine the binding stoichiometry of fatty acid to SCP-2, the relative areas of the fatty acid carboxylate signal were compared. Comparing the highest bound intensity observed between stearate (Figure 1C) to that of oleate (Figure 2C) suggested over a 3 fold higher binding of oleate to SCP-2 than stearate. However, inspection of the relative *areas* between these two fatty acid signals indicated that only 2 equiv of oleate are bound relative to 1 equiv of stearate, in agreement with the known quantity of fatty acid added. Furthermore, the addition of the third equivalent of oleate (Figure 2D) resulted in little change in area of the bound signal despite the dramatic decrease in its intensity due to the broadening of the signal. In addition, the relative areas between the bound and unbound oleate signal after addition of the third equivalent of oleate was found to be approximately 2:1, confirming the complex was already saturated at 2 equiv, the third equivalent remaining completely in the free, unbound form in solution.

Effect of pH on ^{13}C-Fatty Acid:SCP-2 Complexes. Fatty acid labeled at the C-1 position (carboxyl group) serves as sensitive indicator for the ionization state of the fatty acyl chain carboxylate bound to SCP-2. Three results were obtained indicating that the fatty acid was bound to SCP-2 with the hydrophobic methyl end buried deep in the

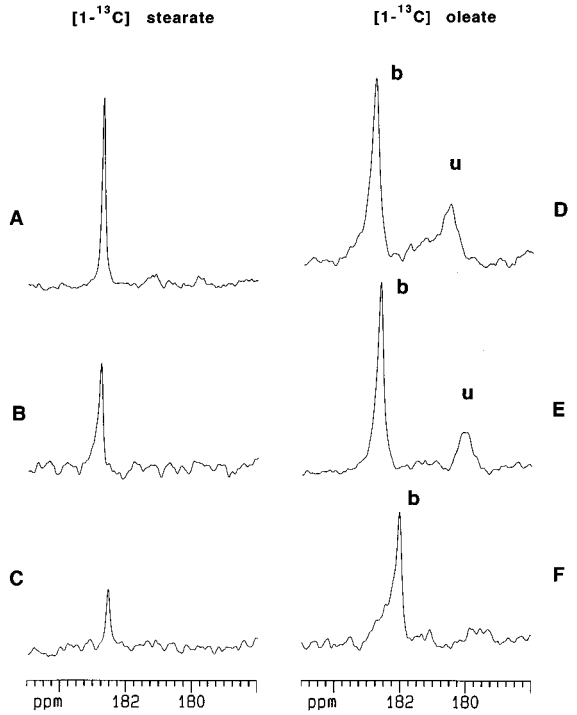

FIGURE 3: The effect of pH upon ^{13}C carboxyl chemical shift and intensity of enriched fatty acid:SCP-2 complexes. Spectra A−C resulted from the 1:1 [1-^{13}C]stearate:SCP-2 complex acquired at pHs 7.38, 7.05, and 6.78, respectively. Spectra D−E resulted from [1-^{13}C]oleate:SCP2 complex obtained at 3:1 mol ratio resulting in bound (B) and unbound (U) forms and acquired at pHs 7.90, 6.98, and 6.30, respectively. All six spectra were scaled equivalently by setting the protein resonances to equal heights.

hydrophobic binding pocket and the fatty acyl chain carboxylate exposed at the surface of the protein. (i) At neutral pH a downfield chemical shift of oleic or stearic acid bound to SCP-2 was observed, reflecting a fully ionized carboxylic acid group. (ii) The addition of dilute HCl (0.1 N) to the [1-^{13}C]stearate:SCP-2 complex further led to a rapid loss of signal intensity (Figure 3A−C) coinciding with increasingly larger amounts of precipitant, indicative of dissolution of the complex resulting in crystalline fatty acid. No detectable signal of the labeled stearate was observed below pH 6.5. The addition of acid to the [1-^{13}C]oleate:SCP-2 complex (2:1), however, resulted in a more complete titration curve before gradual loss of signal intensity occurred. These data do not distinguish between two molecules of oleate binding to two distinct sites, or cooperatively to one site. As shown in Figure 3D−F, approximately 20% loss in signal intensity of the bound [1-^{13}C]oleate had occured by pH 6.3, while at this pH all of the signal of the free, unbound [1-^{13}C]oleate had disappeared. (iii) Addition of dilute HCl (0.1 N) to the fatty acid:SCP-2 complex resulted in an upfield chemical shift of the fatty acyl chain carboxylate (Figure 4A). Assuming a typical value of 4.5−5.0 ppm for the upfield chemical shift upon complete protonation of the carboxylic acid, the endpoint of the titration curve can be estimated, and a pK_a of 5.6 determined for [1-^{13}C]oleate bound to SCP-2. This value is slightly higher than would be expected for simple aliphatic carboxylic acids or that of fatty acid monomers in aqueous solution, which would have an pK_a closer to 5.0. While the observed value is somewhat higher than expected, it still indicates a relative ease of protonation and suggests the carboxylate is exposed to solvent. However, this value is much higher than would be predicted if the

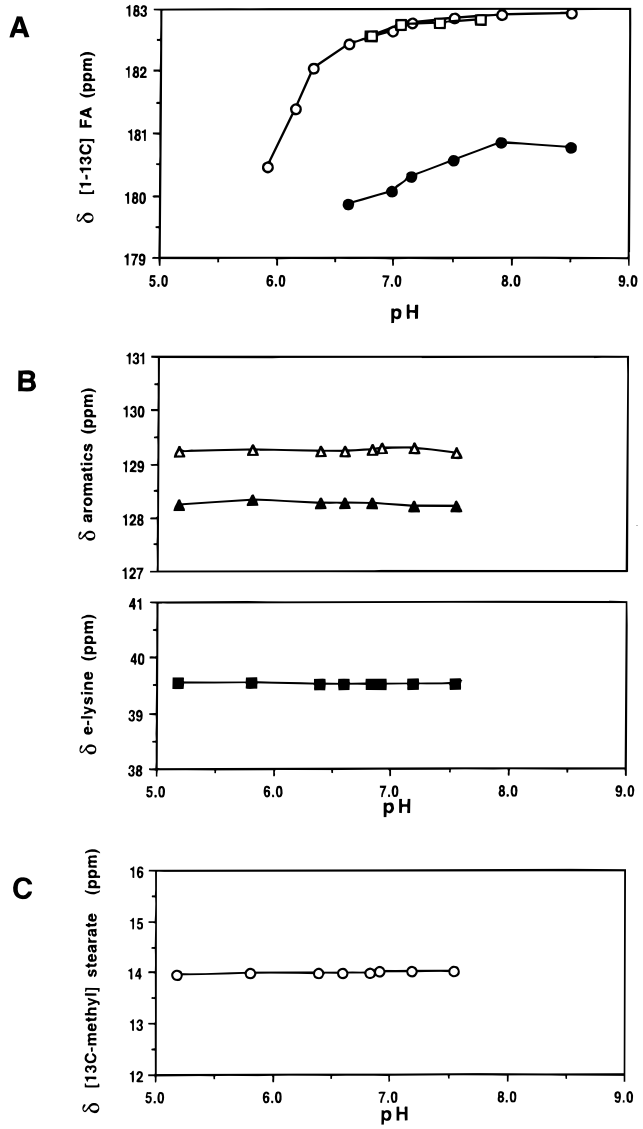

FIGURE 4: pH titration curves of ^{13}C-enriched fatty acid:SCP-2 complexes and selected protein resonances. Panel A: Plot of ^{13}C chemical shift of bound [1-^{13}C]stearate (□), bound [1-^{13}C]oleate (○), and free [1-^{13}C]oleate (●) vs pH. Middle two panels (B): Plot of selected SCP-2 resonances vs pH. Top (△ and ▲), two highest intensity aromatic carbons. Bottom (■), ϵ-lysine carbons. Panel C: Plot of ^{13}C-methyl signal of 1:1 [18-^{13}C]stearate SCP-2 complex vs pH.

carboxylic acid was within a protected environment (e.g., carboxylate buried in the interior of the protein fatty acid binding pocket) or involved in a salt bridge to a protein residue located deep within the binding pocket, as has been observed for another fatty acid binding protein, I-FABP, where an estimated pK_a lower than 4.0 was obtained for [1-^{13}C]palmitate (Cistola et al., 1989, 1990).

In order to conclude that the pH sensitivity of the [1-^{13}C]-fatty acids was due only to the surface exposure of the carboxylate, one must exclude the possibility of a pH-induced global change in SCP-2 structure below pH 7.0 as being responsible for exposure of the fatty acid within the binding pocket and the observed titration of the carboxylate. Two additional experiments were undertaken to resolve this possibility:

First, specific groupings of protein resonances can be monitored for presence of pH-induced chemical shift changes which might be an indication of protein conformation change,

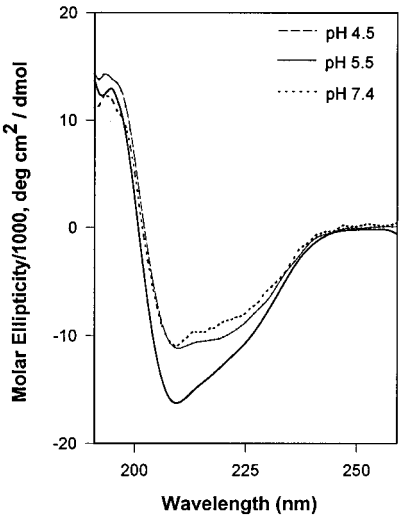

FIGURE 5: pH dependence of circular dichroic spectra of SCP-2. Solid line dotted line, 2 μM SCP-2 in Tris-HCl buffer, pH 7.4; dashed line, 2 μM SCP-2 in Tris-HCl buffer, pH 4.5; solid line, 2 μM SCP-2 in Tris-HCl buffer, pH 5.5. For more details see Materials and Methods.

provided that the specific residue(s) chosen is itself not ionizable in the pH range of interest. The ϵ-lysine carbons ($\delta = 39.45$ ppm) were examined. In the absence of a large pH-induced structural change, the SCP-2 ϵ-lysine carbons should not titrate below pH 9. Likewise, the aromatic carbon region, composed mainly of phenylalanine residues, should also be insensitive to pH. As shown in Figure 4B, no significant changes in either grouping of these protein resonances were noted. This suggests that no large global changes in protein conformation had occured in the pH range 5.5−7.5.

Second, a 1:1 complex of [18-^{13}C]stearate:SCP-2, specifically labeled in the terminal methyl group of the fatty acid, was prepared and titrated as above. The [18-^{13}C] represents the terminal methyl group of stearate. Since the ^{13}C chemical shift for the [18-^{13}C]stearate is totally insensitive to the ionization change of the fatty acid carboxylic acid, it would be an ideal probe to monitor changes of the surrounding protein environment. The result of the [18-^{13}C]stearate (methyl) titration is shown in Figure 4C and confirms that no change in local protein environment occured that was sufficient to alter the methyl group chemical shift.

pH-Dependent Conformational Properties of SCP-2 Studied by Circular Dichroism. As shown above, ^{13}C-NMR spectroscopy did not detect a significant global change in SCP-2 structure upon ligand binding or upon pH titration. This does not, however, rule out the possibility of a localized change in SCP-2 secondary structure upon ligand binding and/or pH titration of the ligand. These possibilities of a secondary structure change in SCP-2 as a function of pH were examined by circular dichroism. CD spectra of the apo-SCP-2 (without bound ligand) were obtained over the pH interval of 4.5−7.4. As shown in Figure 5, the CD spectrum of apo-SCP-2 at pH 7.4 displayed two minima at 208 and 225 nm and one maximum at 193 nm, consistent with the presence of α-helical and β-sheet fractions in the SCP-2 secondary structure. This observation was in a good agreement with NMR data showing the existence of three α-helices and five β-strands in recombinant human SCP-2 secondary structure (Szyperski et al., 1993). Spectrum analysis using the self-consistent method (Sreerama & Woody, 1993) revealed the following proportions of structures in recombinant human SCP-2: 32% α-helix, 21% β-sheet, 25% β-turns, and 22% unordered structure. Addition of ligand oleic acid did not affect the SCP-2 CD spectrum (data not shown). However, decreasing the pH from 7.4 to 6.0−5.0 led to significant alterations of SCP-2 secondary structure, demonstrated mainly by an increase in the peak intensity at 208 nm (Figure 5). Subsequent secondary structure analysis revealed that α-helices and β-sheets were affected the most. Moreover, the nature of the respective pH-dependent changes suggests that an increase in the α-helical component of SCP-2 probably occurred at the expense of the β-sheet component of the protein (Figure 6). Interestingly, at pH 4.5 the shape of the CD spectrum again more closely approached that observed at pH 7.4 (Figure 5) and the proportions of α-helices and β-sheets were much closer to those of SCP-2 at pH 7.4 (Figure 6). Thus, CD data provide additional information on SCP-2 secondary changes. It appears that SCP-2 secondary structure detected by CD is pH sensitive in the range of pH from 4.5 to 7.4.

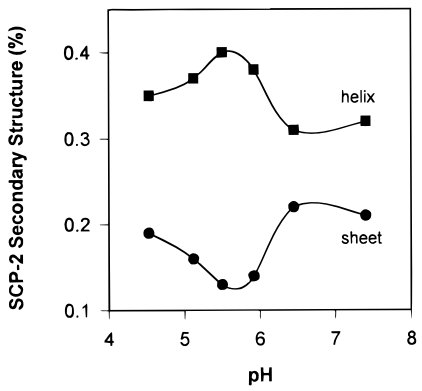

FIGURE 6: Effect of pH on the proportion of α-helical (squares) and β-sheet (circules) structure of SCP-2. For more details see Materials and Methods.

Time-Resolved Fluorescence of Apo-SCP-2 at Different pH. The potential effect of pH on the protein dynamics was further probed by time-resolved fluorescence, using the single Trp residue at position 50. Therefore, the fluorescence of SCP-2 Trp^{50} can be used to monitor rotational dynamics and order of the protein peptide chain in the Trp locus. The results are presented in the Table 2. As seen from the Table 2, no significant alterations were observed in recombinant human SCP-2 Trp^{50} lifetimes, SCP-2 Trp^{50} residual anisotropy r_o, and SCP-2 hydrodynamic radius R upon variation of the buffer pH from 4.5 to 7.4. While overall SCP-2 rotational correlation time, θ near 8.4 ns, decreased at pH below 7, this alteration was not sufficient to alter the SCP-2 hydrodynamic radius R (Table 2). These observations were consistent with decreasing pH altering SCP-2 secondary but not a global tertiary structure. The effect of this pH-induced secondary structure change in SCP-2 on its affinity for a fluorescent fatty acid was determined as described in the following section.

Effect of pH on cis-Parinaric Acid Binding to SCP-2. The possible functional consequences of the pH-induced structural changes of SCP-2 were studied by measuring a binding affinity of recombinant human SCP-2 for *cis*-parinaric acid. Upon titration of SCP-2 with *cis*-parinaric acid, a progressive increase in fluorescence intensity at the emission maximum near 416 nm (Figure 7) was observed. This indicated that

Table 2: pH Effect on Fluorescence Decay and on Anisotropy Decay of Recombinant Human of SCP-2[a]

pH	τ_1	τ_2	α_1	α_2	θ (ns)	r_0	R (Å)
4.5	2.53 ± 0.16	0.79 ± 0.05	0.50 ± 0.01	0.50 ± 0.01	7.10 ± 0.35*	0.169 ± 0.001	20.5
5.9	2.89 ± 0.07	0.87 ± 0.00	0.47 ± 0.001*	0.53 ± 0.001*	7.15 ± 0.20*	0.168 ± 0.002	20.5
7.0	2.85 ± 0.05	0.89 ± 0.02	0.50 ± 0.01	0.50 ± 0.01	8.44 ± 0.14	0.162 ± 0.004	21.0

[a] Values represent the mean (n = 3) ± S.E.M. An asterisk refers to $P < 0.05$ as compared to pH 7.0.

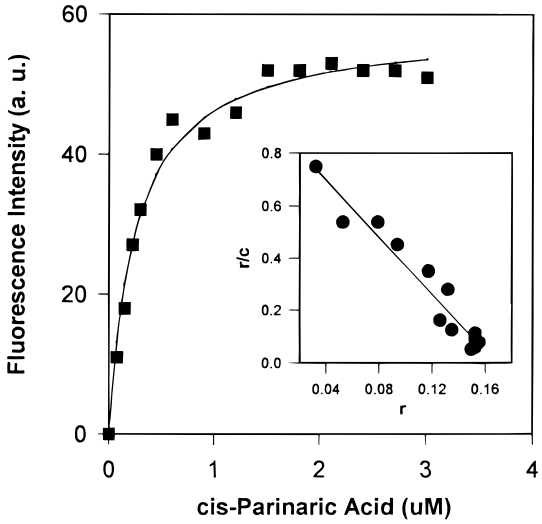

FIGURE 7: Titration of SCP-2 (0.18 μM) with *cis*-parinaric acid (0−3 μM) as followed by the increase in fluorescence intensity. Excitation at 310 nm, emission at 416 nm. Inset: Scatchard plot of the titration of SCP-2 with *cis*-parinaric acid. Buffer pH 4.5.

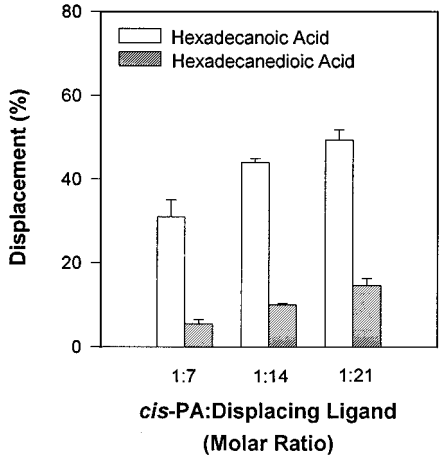

FIGURE 8: Displacement of *cis*-parinaric acid bound to SCP-2 by hexadecanoic and hexadecandioic acid. Protein concentration was 0.18 μM; *cis*-parinaric acid concentration was 0.45 μM. Excitation and emission were at 310 and 416 nm, respectively.

the microenvironment of the fluorophore was less hydrophilic, i.e., it was localized deep in a hydrophobic binding pocket of SCP-2. No shift of fluorescence emission maximum was detected upon titration of SCP-2 with *cis*-parinaric acid (data not shown).

Fluorescence intensities of *cis*-parinaric acid bound to SCP-2 were measured at 416 nm after each addition of ligand, corrected for the background (ligand without protein), and plotted as a function of total ligand concentration . This procedure yielded pure saturation curves for this fatty acid as shown in a representative binding curve (Figure 7). F_{max} parameters were obtained by fitting the saturation curves to a rectangular hyberbola. These results were compared to those obtained from a reverse titration, i.e., when a fixed *cis*-parinaric acid concentration was titrated with the increased amounts of SCP-2 (data not shown). Excellent correlation was found between F_{max} values obtained from direct and reverse titrations. Once the F_{max} value was determined, the concentration of the bound *cis*-parinaric acid could be calculated. This allowed determination of the binding parameters, K_d and B_{max}, as shown by a representative Scatchard plot (Figure 7, inset). SCP-2 had a single binding site for *cis*-parinaric acid with a K_d of 0.65 ± 0.08 μM and a B_{max} of 1.00 ± 0.09 at neutral pH. The same algorithm was employed to measure binding affinities of SCP-2 to *cis*-parinaric acid as a function of pH. The calculated K_d and B_{max} for *cis*-parinaric acid at pH 5.5 were 0.51 ± 0.05 and 0.95 ± 0.01 μM, respectively, not significantly different from those at pH 7. The same values at pH 4.5 were measured to be 0.16 ± 0.01 and 0.98 ± 0.02 μM, respectively. These data allow two observations to be made in describing the pH-dependence of SCP-2 binding of fatty acid: First, over the pH range 7.0−4.5, the SCP-2 has a single binding site. Second, with decreasing pH from 7.0 to 4.5, the SCP-2 binding affinity for *cis*-parinaric acid increased by 4-fold. Thus, the secondary structure change observed at lower pH with CD spectroscopy (see above) was not associated with a decrease in fatty acid binding site number or affinity.

Interaction of SCP-2 with Mono- and Dicarboxylic Fatty Acids. The possible orientation of fatty acid within the SCP-2 binding site was further examined with a displacement assay. *cis*-Parinaric acid bound to SCP-2 was displaced with hexadecanoic acid and with hexadecandioc acid. Addition of increasing C-16 hexadecanoic acid (monocarboxylic) readily displaced up to 55% of SCP-2 bound *cis*-parinaric acid (Figure 8). In contrast, addition of increasing C-16 hexadecanedioc (dicarboxylic) displaced 4−6-fold less SCP-2 bound *cis*-parinaric acid at the equivalent ratios of palmitic: *cis*-parinaric acid ratio of 21:1 (Figure 8). This suggests that the fatty acid can bind within the SCP-2 binding site with the fatty acid carboxyl group oriented either near the surface of the SCP-2 protein molecule or buried deep in the SCP-2 fatty acid binding site. However, the dicarboxylic vs monocarboxylic fatty acid binding data further indicate that SCP-2 more strongly binds fatty acids with the polar carboxyl end oriented at the surface rather than with the fatty acid carboxyl oriented deep in the hydrophobic binding site, consistent with the NMR pH titration data presented above.

DISCUSSION

The observation that sterol carrier protein-2 binds fatty acids (Schroeder et al., 1995) identified SCP-2 as a new fatty acid binding protein. Taken together with its intracellular distribution (Keller et al., 1989), this suggests that future investigations may focus on potential functions of SCP-2 in fatty acid oxidation/metabolism. It also provides a unique opportunity to examine for the first time in depth the properties of the SCP-2 ligand binding site. Fatty acids, unlike other lipid ligands with low critical micellar concen-

tration (cholesterol and phospholipids), have a higher critical micellar concentration, making these observations possible. The aim of the present work was to take advantage of mutually supportive strengths of NMR, CD, steady state fluorescence, and time-resolved fluorescence techniques to obtain new insights regarding the fatty acid binding site of SCP-2.

NMR techniques demonstrate that common naturally occurring fatty acids such as oleic and stearic acid bind to SCP-2. NMR can monitor fatty acid binding without separating bound from unbound fatty acid. Furthermore, the ^{13}C nucleus is a nonperturbing probe, a problem observed with many fluorescent-labeled and spin-labeled fatty acids. The binding of several ^{13}C-enriched long chain fatty acids to human SCP-2 was investigated by ^{13}C-NMR spectroscopy. The observed chemical shifts of 182.7 and 180.8 ppm for ^{13}C-labeled fatty acids are in good agreement with those observed for L-FABP (Cistola et al., 1988), which were assigned to bound and unbound forms of oleate, respectively. Even at concentrations used for NMR spectroscopy (1−2 mM), SCP-2 at neutral pH was found to bind 1−2 equiv of C-18 fatty acids despite limited or poor solubility of the fatty acid at that concentration. SCP-2 readily bound 1 equiv of stearate, even though the fatty acid is practically insoluble in aqueous buffer at 1 mM. These results confirm earlier studies with fluorescent fatty acids (Schroeder et al., 1995) that SCP-2 binds various long chain fatty acids with high affinity. In the case of oleate binding, SCP-2 was shown to bind up to 2 equiv of [1-^{13}C]oleate, before unbound oleate was detected in solution by NMR. This suggests that the binding affinity of SCP-2 for oleate was sufficiently strong so that unbound oleate was not detected until saturation of the protein was achieved, only after which does additional oleate become available free insolution and undergoes slow exchange with bound oleate. An additional fatty acid binding site was observed by NMR with [1-^{13}C]oleate than with the binding of fluorescent 'kinked chain' analogue of oleate, i.e., *cis*-parinaric acid data. This is most likely due to the 10^3−10^4 times higher fatty acid concentration used in the NMR vs the fluorescence binding assay, i.e., the NMR experiment reveals the existence of an additional binding low affinity site not detected by fluorescence techniques using significantly lower fatty acid concentration. A similar situation was observed with NMR studies of fatty acid binding to L-FABP where a higher than expected stoichiometry was present (Cistola et al., 1988). In summary, the NMR studies clearly show that SCP-2 binds fatty acids, thereby confirming earlier studies with fluorescent fatty acids (Schroeder et al., 1995). Four types of studies were consistent with the bound fatty acid being oriented in the binding site with the fatty acid carboxylate exposed near the surface of the protein:

First, the NMR chemical shift (182.7 ppm) and narrow line width (<20 Hz) of the C-1 carbonyl of the fatty acid: SCP-2 complex above pH 7.0 is consistent with fully ionized monomeric carboxylate anion, very similar to that found for fatty acid complexes of L-FABP (Cistola et al., 1989), described as being consistent with a highly solvated binding environment (Gurd & Keim, 1973; Maciel & Traficante, 1960; Cistola et al., 1988).

Second, the ionization of fatty acid carboxylate when the fatty acid was bound to SCP-2 supports high solvent accessibility of the carboxylate terminus of the fatty acid. The estimated pK_a for the protonation of oleate bound to SCP-2 was approximately 5.5.

Third, the dicarboxylic C-16 fatty acid, hexadecandioic acid, was 6-fold less effective in displacing SCP-2 bound *cis*-parinaric acid than was the monocarboxylic C-16 fatty acid, hexadecanoic acid. Accordingly, these results are highly suggestive that SCP-2 binds the lipophilic tail (methyl end) of the fatty acid within a hydrophobic binding site, leaving the ionized carboxylate near the suface of the protein, readily accessible to solvent protonation.

Fourth, the pH-dependent dissociation behavior of oleic acid bound to SCP-2 supports high solvent accessibility of the carboxylate terminus of the fatty acid. The SCP-2: stearate complex more readily dissociated below pH 7 (which in itself, indicates a high degree of solvent accesibility), and its initial titration curve (Figure 4A) matches that of the oleate complex. Similar results with the intestinal fatty acid binding protein, I-FABP, also indicate that if the fatty acid carboxylate is buried in the binding protein interior, it must not be accessible to pH titration (Cistola et al., 1989, 1989, 1990). The alternate possibility that these results might be explained by SCP-2 undergoing a global and/or secondary change in structure resulting in lowered binding affinity for fatty acid was excluded. To preclude pH-induced changes in SCP-2 global structure, NMR chemical shifts of three specific groups of resonances (two from the protein, and one additional resonance from the ligand) were examined. These chemical shifts of these groups were not altered by decreasing pH. In the former case, bulk resonances associated with protein lysines (ϵ-carbon) and the aromatic carbons of phenylalanine were found to be unaffected by pH over the range 5−7.5. While each group of resonances represents the sum of many individual carbons, qualitatively, the result can be intepreted that no large global structue change in SCP-2 structure occurred with decreasing pH. This finding parallels that observed for L-FABP, the only other cytosolic fatty acid binding protein in which the fatty acid is also oriented with the carboxylate facing the surface (Cistola et al., 1988, 1989, 1990). At pH below 7, L-FABP showed displacement of fatty acid, CD changes, and no changes in global structure as detected by NMR. Finally, time-resolved fluorescence spectroscopy of SCP-2 Trp^{50} showed that neither the SCP-2 Trp^{50} lifetime, SCP-2 Trp^{50} residual anisotropy, nor SCP-2 hydrodynamic radius were sensitive to lowered pH, again consistent with the absence of global structural changes in SCP-2. Although the overall rotational rate of SCP-2 decreased slightly at lower pH and CD indicated that SCP-2 underwent changes in secondary structure at lower pH, these did not result in lower affinity or lower stoichiometry of *cis*-parinaric acid binding to SCP-2 over the range pH 7.4−4.5. There may, however, be problems in relating the conformational changes observed at micromolar concentrations in the CD experiments with the lack of conformational changes observed at millimolar concentrations used in the NMR experiments. It should be noted that cellular fatty acid and SCP-2 concentrations are in the micromolar range 20 μM (Glatz et al., 1993) and 4−40 μM (Gossett et al., 1996), respectively.

The location of the SCP-2 change in secondary structure (increased α-helix) detected by CD of SCP-2 at lower pH is not known, but some inferences may be made. The CD studies presented herein as well as previous NMR investigations (Szyperski et al., 1993) indicate that the main structural features of SCP-2 are a relatively large amino terminal

amphipathic α-helix, a large amount of β-sheets, and smaller α-helix structures near the carboxy terminal. The N-terminal α-helix, the single Trp^{50}, and a putative ligand binding site near the C-terminal appear to be relatively distant from one another. For the following reasons it appears likely that the observed CD changes in SCP-2 secondary structure at lower pH were in the vicinity of the α-helix regions, perhaps near the putative C-terminal ligand binding site. (i) The affinity of SCP-2 for fatty acid increased at lower pH. However, fatty acid binding in itself did not significantly affect the secondary structure of SCP-2 detected by CD and by the lack of chemical shift of the ^{13}C-labeled methyl group of SCP-2 oleate as described herein and elsewhere (Frolov et al., 1996a). (ii) The location of the secondary structure change within SCP-2 appears to be distant from the SCP-2 Trp^{50}. The absence of significant changes in the SCP-2 Trp^{50} lifetimes, residual anisotropy, and radius as a function of pH suggest that the secondary structure changes detected by CD were not near the vicinity of Trp^{50} of SCP-2. Several studies suggest that Trp^{50} residue may also be far removed from the ligand binding site: The NMR secondary and three-dimensional structure of SCP-2 shows that Trp^{50} is located on the opposite side of the putative ligand binding site of SCP-2 (Szyperski et al., 1993). Structure activity studies were consistent with this ligand binding site being located near the SCP-2 carboxyl terminus. Deletions or mutations in the carboxyl terminus of SCP-2 inhibited the sterol transfer activity of the SCP-2 (Seedorf et al., 1994). Forster nonradiative energy transfer studies showed that the SCP-2 Trp^{50} is located 33−40 Å from the *cis*-parinaric acid binding site of SCP-2 (Frovlov & Schroeder, unpublished results).[3] Thus, while changes in SCP-2 seondary structure occur as a function of decreased pH, these changes appear removed from the SCP-2 Trp but near the fatty acid binding site (increased affinity for fatty acid at low pH). Future investigations will need to determine if the free fatty acid binding site of SCP-2 is identical to the cholesterol binding site.

The substantial pH-induced change in SCP-2 secondary structure detected by CD and the smaller change in SCP-2 overall rotational rate may relate to the mechanism whereby SCP-2 enhances intermembrane ligand transfer. Because fatty acids, fatty acyl CoAs, and other SCP-2 ligands (cholesterol and phospholipids) are localized primarily to membrane structures, the proportion of these ligands found in the cytosol is low [reviewed in Glatz et al. (1993); Gossett et al., (1996)]. It is interesting to note that, in contrast to native and recombinant FABPs, SCP-2 contains very little endogenously bound fatty acid. Native and recombinant FABPs (Paulussen & Veerkamp, 1990) have nearly half of their binding sites occupied by endogenous fatty acid. The basis for this difference is not known but may involve the very different isolation procedures used to obtain SCP-2 as compared to FABPs or may be due to the very low concentration of cellular free fatty acid. While cellular total fatty acid concentration is 20 μM, the majority of fatty acid is membrane bound such that the cytosolic fatty acid concentration is only 2 nM (Glatz et al., 1993). One function of lipid transfer proteins such as SCP-2 is to enhance the desorption and/or transfer of these lipid ligands between membranes (Wirtz, 1991; Moncecchi et al., 1991a; Paulussen & Veerkamp, 1990). For example, SCP-2 is a cationic protein (pI > 8.5) that requires an intact ligand binding site and interacts directly with membranes to bind and/or enhance intermembrane ligand transfer (Woodford et al., 1995). SCP-2 enhances intermembrane ligand transfer several orders of magnitude faster when the membrane contains anionic phospholipids, conferring a negative charge to the lipidic surface (reviewed in (Schroeder et al., 1991, 1996). The hydrogen ion concentration at the membrane/aqueous interface, especially in the presence of anionic phospholipids, is several orders of magnitude higher than in the bulk aqueous medium at pH 7 (Tocanne & Teissie, 1990). What effect of such a low interfacial pH at the membrane surface has on the ability of SCP-2 to bind to the membrane and/or interact with ligand is not known. What is known, however, is that at pH below 7, SCP-2 underwent a change in secondary structure as evidenced by CD spectra showing increased α-helix and decreased β-sheets. NMR data show that SCP-2 contains an amino terminal amphipathic (basic) α-helix and also some smaller carboxyl terminal α-helices (Szyperski et al., 1993) that are essential to the ligand transfer function of this protein (Seedorf et al., 1994). The much higher proton concentration (lower pH) at the membrane surface (vs bulk aqueous phase pH) may lead to increased α-helix as observed herein for SCP-2 at low pH. An increase in α-helix may promote the interaction of the SCP-2 with the membrane surface. Although interesting, there is as yet no definitive proof for this possibiltiy. Alternately, an increase in the proportion of α-helix may promote the interaction of the membrane bound SCP-2 with membrane bound ligand. Consistent with the latter possibility, the affinity of SCP-2 for *cis*-parinaric acid at low pH was 4-fold higher than at neutral pH. These differences in SCP-2 structure and function at lower pH may be important in view of the reported intraperoxisomal matrix pH being 5.8−6.0 (Nicolay et al., 1987).

In summary, NMR, CD, steady state fluorescence, and time-resolved fluorescence data show that human recombinant SCP-2 has a fatty acid binding site in which the fatty acid is oriented with its carboxylate group exposed to the aqueous solvent. SCP-2 is a basic protein (pI = 8.5) that shows no significant sequence identity/homology and little structural similarity to members of the fatty acid binding protein family (FABPs, pI values generally below 7) (Pfeifer et al., 1993a; Wirtz, 1991a; Moncecchi et al., 1991a; Vahouny et al., 1987a). Nevertheless , SCP-2 binds fatty acids with similar affinity and with a 1:1 stoichiometry as has been observed for most of the FABPs (Banaszak et al., 1994; Paulussen & Veerkamp, 1990; Bass, 1985; Schroeder et al., 1993). As shown herein, SCP-2 and L-FABP (Banaszak et al., 1994) may be unique among the cytosolic fatty acid binding proteins in that they both bind the fatty acid with the fatty acid carboxylate exposed at the surface of the protein. Finally, the detailed NMR, fluorescence, and CD analysis of the SCP-2 fatty acid binding site as described herein appears physiologically relevant and provided additional insights into possible mechanism(s) whereby this protein may interact with ligands such as fatty acids to enhance intermembrane ligand transfer.

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