Regulation of Membrane Cholesterol Domains by Sterol Carrier Protein-2†

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ABSTRACT: Sterols are not randomly distributed in membranes but appear to be localized in multiple kinetic domains. Factors that regulate these sterol domains are not well-understood. A recently developed fluorescence polarization assay that measures molecular sterol transfer [Butko, P., Hapala, I., Nemecz, G., of Schroeder, F. (1992) *J. Biochem. Biophys. Methods 24*, 15–37] was used to examine the mechanism whereby anionic phospholipids and liver sterol carrier protein-2 (SCP_2) enhance sterol transfer. Two exchangeable and one very slowly or nonexchangeable sterol domain were resolved in phosphatidylcholine (POPC)/sterol small unilamellar vesicles (SUV). Inclusion of 10 mol % anionic phospholipids enhanced sterol exchange primarily by redistribution of sterol domain sizes rather than by alteration of half-times of exchange. This effect was dependent primarily on the percent content rather than the net charge per anionic phospholipid. In contrast, SCP_2 simultaneously altered both the distribution of sterol molecules between kinetic domains and the exchange half-times of exchangeable sterol domains. The effects of SCP_2 were much more pronounced when 10% acidic phospholipid was incorporated in the SUV. Compared to spontaneous sterol exchange, in the presence of 1.5 μM SCP_2, the rapidly exchanging pool was increased by 36 to 330%, depending on the SUV phospholipid composition. Concomitantly, exchange half-times for rapidly and slowly exchangeable sterol were reduced by 60 to 98% for $^1t_{1/2}$ and 14 to 85% for $^2t_{1/2}$, respectively. The stimulatory effect of SCP_2 was saturable and dependent both on protein concentration and on content of acidic phospholipids in membranes. The effect of acidic phospholipids on SCP_2 activity could be reduced by screening of membrane surface charge with mono-, di- and, polyvalent cations. This indicated the involvement of ionic interactions between the protein and membrane in SCP_2 action. The effect of SCP_2 on sterol-exchange kinetics cannot be explained solely by its ability to bind sterols as other sterol-binding protein (liver fatty acid binding protein) did not stimulate sterol exchange. The results indicate that the mechanism of SCP_2 action involves both changes in membrane domain structure (relative sizes of sterol kinetic pools) and changes in the membrane–water interface (sterol desorption rate) induced upon interaction of protein with the membrane surface.

Intracellular sterol distribution is in sharp contrast with the distribution of sterol synthetic activities. The majority of intracellular sterol is located in the plasma membrane while the membranes most active in sterol synthesis (endoplasmic reticulum, mitochondria) have the lowest sterol content in the cell. There must therefore be extensive flux of sterol molecules between intracellular membranes (Schroeder et al., 1991; Wirtz & Gadella, 1990; Reinhart, 1990; Dawidowicz, 1987). Although sterol molecules were shown to cross the aqueous space between membranes by simple diffusion of monomers *in vitro* (McLean & Phillips, 1981; Backer & Dawidowicz, 1981), this mechanism is not likely to explain the nonequilibrium intracellular distribution of sterols in intact cells. Both vesicle trafficking (Lange & Steck, 1985; Urbani & Simoni, 1990; Kaplan & Urbani, 1985) and specific hydrophobic ligand-binding proteins (Schroeder et al., 1991; Wirtz & Gadella, 1990; Reinhart, 1990; Dawidowicz, 1987) could contribute significantly to the establishment and maintenance of the asymmetric intracellular sterol distribution in eukaryotic cells.

The hypotheses on direct role(s) of specific cytosolic proteins *in vitro* on intermembrane sterol transfer were deduced from the ability of these proteins to stimulate specific reactions of sterol metabolism (Noland et al., 1980, Gavey et al., 1981; Trzaskos & Gaylor, 1983) and to enhance sterol transfer between membranes (Trzaskos and Gaylor, 1983; Muczynski & Stahl, 1983; Chanderbhan et al., 1981; Van Amerongen et al. 1989; Butko et al. 1990, Schroeder et al., 1990a). Although one of these proteins, SCP_2[1] (also called nonspecific lipid transfer protein, ns-LTP), has been studied extensively, the mechanism whereby it enhances lipid transfer is still not clear (see Vahouny et al., 1987, for review). SCP_2 was shown *in vitro* to bind phospholipids (Nichols, 1987; Gadella et al.,

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[1] Abbreviations: Sterol carrier protein-2, SCP_2; liver fatty acid binding protein, L-FABP; sterol carrier protein, SCP.

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1991) and sterols (Schroeder et al., 1990b). However, simply binding a specific ligand like cholesterol does not necessarily correlate with function (Billheimer & Reinhart, 1991) and may simply be coincidental as in the case of elastase (Sziegoleit, 1982). Additional evidence obtained *in vitro* indicates that SCP_2 enhances the intermembrane transfer of various phospholipids (Bloj & Zilversmit, 1977) and cholesterol (review, Schroeder et al., 1991; Wirtz & Gadella, 1990; Billheimer & Reinhart, 1991). Nevertheless, there is little agreement regarding either the function of SCP_2 in the cell or its molecular mechanism(s) of action. It is not even clear whether this protein is more specific for a particular lipid class, e.g. cholesterol (Schroeder et al., 1990), or if it is really a "nonspecific" lipid carrier.

The present investigation was undertaken to examine the mechanism whereby sterol exchange between small unilamellar vesicles (SUV) is enhanced by SCP_2. In order to avoid the potential pitfalls of separation assays, a recently developed fluorescence polarization method (Nemecz & Schroeder, 1988; Nemecz et al., 1988) that enables a continuous analysis of sterol-exchange kinetics without prior separation of donor and acceptor membranes was used. The assay utilizes fluorescent dehydroergosterol ($\Delta^{5,7,9(11),22}$-ergostatetraene-3β-ol) as a sterol probe. This sterol does not contain any bulky side groups and the conjugated triene fluorophore is native to the molecule (Nes et al., 1989; Schroeder et al., 1991). Structural and dynamic properties of dehydroergosterol in membranes are very similar to those of cholesterol (review, Schroeder et al., 1991; Schroeder & Nemecz, 1990; Schroeder, 1984) such that it can replace over 80% of native sterol in cultured fibroblasts (Hale & Schroeder, 1981), it binds to sterol carrier proteins similarly as radiolabeled cholesterol (Nemecz & Schroeder, 1991; Schroeder et al., 1990; Fischer et al., 1985), and it behaves nearly identically to cholesterol in exchange assays (Nemecz et al., 1988). Thus, dehydroergosterol provides a useful probe for analysis of sterol-domain structure and kinetics of sterol exchange.

It was shown in previous studies (Butko et al., 1990; Schroeder et al., 1990a; Billheimer & Gaylor, 1990) that the initial rate of sterol exchange between model membrane vesicles could be stimulated up to 40-fold in the presence of SCP_2. These earlier studies did not, however, address the effects of SCP_2 on specific sterol domains in membranes. Multiple sterol domains were recently identified in phase diagrams of model membranes and from static and kinetic analyses of sterols in both model and biological membranes (review, Schroeder et al., 1991; Mahlberg & Rothblat, 1992; Bar et al., 1987, 1989). Herein, we have reexamined the enhancement by SCP_2 of sterol-transfer rates using a more detailed kinetic analysis which consisted of the correction for acceptor polarization values related to light scatter from the signal and proper calibration of the initial polarization values to those of the standard curve. This technical progress improved the resolution primarily of the size of the nonexchangeable sterol domain compared to previous reports. The results demonstrate clearly that the strong effect of SCP_2 on sterol initial rate of exchange is mediated by shifts in the distribution of sterol molecules between intramembrane sterol kinetic domains as well as by decrease in exchange half-times corresponding to these domains.

MATERIALS AND METHODS

Materials. 1-Palmitoyl-2-oleoylphosphatidylcholine (PC) and bovine heart cardiolipin (CL) were obtained from Avanti Polar Lipids, Inc. (Birmingham, AL). Cholesterol was from Applied Science Laboratories, Inc. (State College, PA). Bovine brain L-α phosphatidyl-L-serine (PS), bovine liver L-α-phosphatidylinositol (PI), and neomycin sulfate were purchased from Sigma Chemical Co. (St. Louis, MO). Dehydroergosterol was synthesized and purified as described previously (Fischer et al., 1985b; Fischer et al., 1984). All sterols were recrystallized in ethanol and their purity was checked by HPLC prior to use. SCP_2 was purified from rat liver according to Noland et al. (1980).

Vesicle Preparation. Small unilamellar vesicles (SUV) composed of PC/sterol or PC/acidic phospholipid/sterol (molar ratios 65:35 or 55:10:35, respectively) were prepared as described earlier (Schroeder et al., 1987) with some modifications: (i) The buffer used for vesicle preparation (10 mM PIPES/0.02% NaN_3, pH 7.4) was prefiltered through a 0.2-μm filter (Millipore, Bedford, MA). In some experiments the buffer contained 0.15 M KCl. (ii) The sonication time for preparation of SUV with and without acidic phospholipids was 2.5–4 and 30 min, respectively.

Sterol-Exchange Assay. The exchange of dehydroergosterol for cholesterol was monitored by an assay based on the method of Nemecz et al. (1988) modified to determine molecular sterol transfer (Butko et al., 1992). The final lipid concentration in the exchange assay was maintained at 150 μM with 10-fold excess of acceptor over donor SUV. The vesicle composition was the same for donors and acceptors except for the nature of the sterol (35 mol % of dehydroergosterol or cholesterol in donor and acceptor vesicles, respectively). Lyophilized proteins were dissolved in sterile distilled water prior to use. During the exchange assay the sample was continuously stirred at 24 °C using a resident micro stir bar (Fisher Scientific Inc., Pittsburgh, PA) in the cuvette. The extent of sterol transfer was monitored by continuous measurement of dehydroergosterol polarization for 3–5 h using an SLM 4800 spectrofluorometer (SLM Instruments, Inc., Urbana, IL) in the T-format. Data were collected automatically every 18 s by a Compaq PC computer through an ISS-ADC interface. The light source was a 450 W xenon-arc lamp. The excitation and emission wavelengths were set to 325 and 376 nm, respectively. The inner-filter effect and light scattering were made negligible by using dilute vesicle suspensions (absorbance at 325 nm <0.1) and by placing GG-375 sharp cutoff filters (Janos Technology, Inc., Towsend, VT) in the emission system.

Turbidity (Light-Scatter) Measurements. Light scattering at 325 nm was measured at 90° in the above SLM 4800 spectrofluorometer without emission monochromator or emission cutoff filters. The lipid concentration in the cuvette was maintained at 150 μM, which was the concentration used in the exchange experiments.

Data Analysis. Detailed information regarding the biophysical and mathematical basis of the fluorescence polarization method for measuring sterol exchange from dehydroergosterol polarization is provided elsewhere (Nemecz & Schroeder, 1988; Butko et al., 1992; Woodford et al., 1993). The method is based on the observation that in model membranes the polarization of the light emitted by dehydroergosterol is dependent on dehydroergosterol concentration in the membrane (Nemecz et al., 1988). The dependence of polarization, P, on dehydroergosterol concentration can be described in standard curves for vesicle membranes with the following polynomial function:

$$P = mx_d^2 + n \quad (1)$$

where x_d is the dehydroergosterol concentration in donor vesicles, and the parameters m and n ($m = 0.1673$ and $n = 0.3235$ for all vesicles used herein) are obtained using computer nonlinear regression. The complete derivation of this equation is presented elsewhere (Butko et al., 1992).

Earlier results from this laboratory using model membrane systems suggested that sterol exchanges with biexponential kinetics (Nemecz et al., 1980; Nemecz & Schroeder, 1988; Butko et al., 1992; Woodford et al., 1993) described by the following equation:

$$X_d = A_2 \exp(-k_{E1}t) + B_2 \exp(-k_{E2}t) + C_2 \quad (2)$$

When eq 2 is inserted into eq 1, we obtain:

$$P = -m[A_2^2 \exp(-2k_{E1}t) + 2A_2B_2 \exp(-k_{E1}t - k_{E2}t) + B_2^2 \exp(-2k_{E2}t) + 2A_2C_2 \exp(-k_{E1}t) + 2B_2C_2 \exp(-k_{E2}t) + C_2^2] + n \quad (3)$$

where A_2, B_2, and C_2 represent the two exchangeable and the nonexchangeable sterol fractions, respectively, k_{E1} and k_{E2} represent the corresponding rate constants of the exchangeable fractions, and the constants m and n are those from eq 1. Equation 3 was used in the fitting of experimental curves with four fitted parameters, k_{E1}, k_{E2}, A_2, and B_2. The value of C_2 must satisfy the condition that $A_2 + B_2 + C_2 = 1$. The above biexponential equation fits the exchange data better than the monoexponential as indicated by significantly reduced values of χ^2. Equation 3 was used for calculating kinetic parameters. In contrast to earlier work (Nemecz & Schroeder, 1988; Butko et al., 1992), the data from each experiment were corrected for light scatter. Light-scattering contribution to the polarization signal was reduced by use of dilute SUV concentration, appropriate cutoff filters, and, where necessary, by subtraction of polarized blank vesicle (without dehydroergosterol) intersites. This resulted in initial polarization values which were properly calibrated to those of the standard curve, which significantly improved the sterol kinetics determination. Multiexponential curve fitting to the 600–1000 polarization data points obtained per exchange was performed on a VAX 8650 computer (Digital Equipment Corp., Maynard, MA) with a NLIN nonlinear-least-squares-regression procedure (SAS Institute, Inc., Cary, NC). Curve fittings to experimental data used for derivation of empirical equations (see following section) were carried out with Graphpad software (ISI, Philadelphia, PA) on an IBM PC.

RESULTS

Effect of SUV Lipid Composition on Membrane Sterol Domains: Spontaneous Sterol Exchange. Mixing of donor SUV containing fluorescent dehydroergosterol with acceptor vesicles of the same phospholipid composition containing cholesterol instead of dehydroergosterol resulted in exchange of sterol molecules between donor and acceptor membranes. What was observed, in fact, was the continuous dilution of exchanged dehydroergosterol in 10-fold excess of cholesterol. This process was manifested as a continuous increase in dehydroergosterol fluorescence polarization (Figure 1). In the absence of acceptors, dehydroergosterol polarization was stable in PC/sterol (Figure 1A, curve 1) and in PS/PC/sterol (Figure 1B, curve 1) donor SUV over the time frame of the experiment. Upon addition of acceptor SUV (arrow in Figure 1), dehydroergosterol polarization continuously increased, the effect being more rapid in PS containing SUV (Figure 1B, curve 2, vs 1A, curve 2).

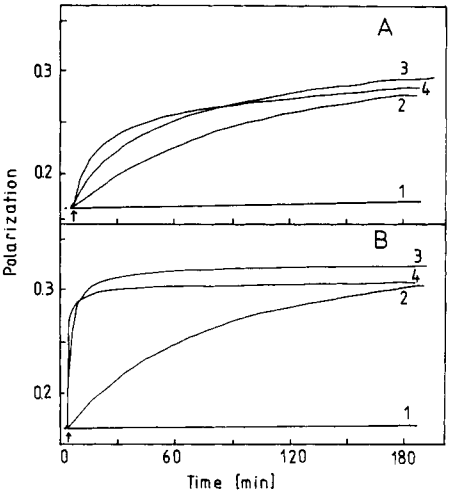

FIGURE 1: Effect of SCP_2 on dehydroergosterol fluorescence polarization change during sterol exchange. Donor SUV of various phospholipid composition containing 35 mol % of dehydroergosterol were mixed with a 10-fold excess of acceptor SUV of the same composition containing 35 mol % of cholesterol at time indicated by the arrow. The exchange was performed at conditions described in Materials and Methods (10 mM Pipes, pH 7.3–0.02% sodium azide; final lipid concentration 150 μM; assay temperature, 24 °C). Curves: 1, donor SUV only; 2, donor and acceptor SUV, no SCP_2; 3, as 2, in the presence of 1.5 μM SCP_2 added together with acceptors; 4, as 3, SCP_2 concentration 10 μM; A, POPC/sterol (65:35) SUV; B, PS/POPC/sterol (10:55:35) SUV.

In order to resolve the exchange kinetics into individual components representing molecular sterol transfer, the kinetic analysis described in Materials and Methods was applied to the polarization data. This treatment of polarization data resulted in conversion of the time-dependent increase in fluorescence polarization directly to molecular sterol flux between donor and acceptor vesicles and in resolution of the kinetics of the exchange process. The data for POPC/sterol SUV displayed biexponential kinetics that would best be described by the following parameters: three sterol fractions (domains) and corresponding exchange half-times for two of them. In the absence of added protein, the spontaneous exchange half-time of the rapidly exchangeable fraction (f_1) was near 27 min while the exchange half-time for the second slowly exchangeable fraction (f_2) was much longer, up to 145 min in spontaneous exchanges (Table 1). The corresponding fractions f_1 and f_2 were 0.11 and 0.70, respectively. The third fraction ($f_3 = 0.21$) can be considered as nonexchangeable as its exchange half-time was so long that it was not resolvable in the time scale of the experiment.

The effect of phospholipid composition on spontaneous sterol exchange was examined in experiments in which donor and acceptor SUV phospholipid composition was maintained the same (Tables 1 and 2). POPC is a zwitterionic phospholipid with net neutral charge. Inclusion of phosphatidylethanolamine, PE, another phospholipid with net neutral charge at pH 7.4, into both donor and acceptor SUV did not alter any of the sterol-exchange kinetic or domain parameters (Table 1). In contrast, inclusion of 10% anionic phospholipids (PS, PI, CL) dramatically altered sterol domain size (fractions) and in some cases sterol-exchange half-times (Table 2 vs Table 1). Inclusion of phosphatidylserine or cardiolipin in the SUV significantly increased the size of the rapidly exchangeable sterol domain f_1 from 0.11 to 0.32 ($p < 0.05$) and 0.21 ($p < 0.05$), respectively (Table 2 vs Table 1). In contrast, only PS significantly increased the $^1t_{1/2}$ to 54 min (Table 2) as compared to 27 min for PC/sterol SUV (Table 1). Thus, the presence of anionic phospholipids stimulated spontaneous sterol

Table 1: Effect of Sterol-Binding Proteins on Sterol-Exchange Kinetics of Neutral Phospholipids[a]

SUV	protein	f_1	f_2	f_3	$^1t_{1/2}$ (min)	$^2t_{1/2}$ (min)
PC (11)	none	0.11 ± 0.04	0.70 ± 0.10	0.21 ± 0.02	27 ± 6	145 ± 19
PC (3)	SCP-2	0.15 ± 0.01	0.48 ± 0.01*	0.37 ± 0.01*	11 ± 1*	93 ± 3*
PC (5)	L-FABP	0.13 ± 0.04	0.59 ± 0.03	0.28 ± 0.04	30 ± 6	138 ± 26
PE (3)	none	0.08 ± 0.03	0.72 ± 0.12	0.21 ± 0.08	28 ± 12	161 ± 35
PE (3)	SCP-2	0.18 ± 0.01*	0.57 ± 0.09	0.25 ± 0.10	10 ± 1	139 ± 35
PE (1)	L-FABP	0.16	0.74	0.09	20	120

[a] Exchange conditions were as described in Figure 1. SUV were composed of 10 mol % of the corresponding phospholipid (PC, PE):55 mol % of PC:35 mol % of sterols (dehydroergosterol in donor, cholesterol in acceptor vesicles). Polarization data treatment and calculations of kinetic parameters are described in Materials and Methods section. The number of independent experiments is shown in parentheses. Values shown in the table are averages ± SEM. An asterisk refers to $p < 0.05$ as compared to no SCP_2.

Table 2: Effect of Sterol-Binding Proteins on Sterol-Exchange Kinetics of Negatively Charged Phospholipids[a]

SUV	protein	f_1	f_2	f_3	$^1t_{1/2}$ (min)	$^2t_{1/2}$ (min)
PS (6)	none	0.32 ± 0.08	0.55 ± 0.11	0.14 ± 0.06	54 ± 11	138 ● 32
PS (6)	SCP-2	0.53 ± 0.005**	0.20 ± 0.007*	0.25 ± 0.01**	1 ± 0.1**	21 ± 3**
PS (2)	L-FABP	0.26 ± 0.02	0.71 ± 0.05	0.04 ± 0.02	56 ± 1	447 ± 22*
PI (2)	none	0.17 ± 0.08	0.76 ± 0.02	0.09 ± 0.06	31 ± 10	155 ± 40
PI (4)	SCP-2	0.48 ± 0.03*	0.19 ± 0.01**	0.32 ± 0.03**	0.7 ± 0.1**	30 ± 6**
PI (1)	L-FABP	0.09	0.62	0.38	17	110
CL (3)	none	0.21 ± 0.02	0.58 ± 0.02	0.21 ± 0.03	27 ± 6	145 ± 19
CL (3)	SCP-2	0.51 ± 0.05**	0.15 ± 0.02**	0.32 ± 0.07	0.75 ± 0.05**	83 ± 35
CL (2)	L-FABP	0.12 ± 0.03	0.70 ± 0.03	0.19 ± 0.06	16 ± 3	116 ± 4

exchange primarily by modulating membrane sterol domain size rather than by decreasing individual half-times of exchange. There was no apparent pattern associated with the presence of two negative charges on the phospholipid (CL) versus one negative charge (PS, PI).

Sterol Carrier Protein-2 Modulates Sterol Domains in SUV Membranes: Potentiation by Anionic Phospholipids. The addition of SCP_2 to the sterol exchange assay mixture dramatically modulated the kinetics of polarization change (Figure 1). It must be considered that the observed changes in the kinetics of polarization change in the presence of SCP_2 could reflect some artefactual polarization shifts due to dehydroergosterol–SCP_2 binding. This possibility was excluded by the demonstration that addition of SCP_2 (up to 4.5 μM) in the absence of acceptor SUV did not affect fluorescence intensity or polarization of donor vesicles (data not shown). The enhancement of sterol exchange by SCP_2 was dependent on SUV composition. The rate of polarization change in the presence of the protein was much slower in POPC/sterol vesicles (Figure 1A, curve 3) than in vesicles containing 10 mol % PS (Figure 1B, curve 3). The above polarization curves were resolved into individual components representing molecular sterol transfer by the kinetic analysis described in Materials and Methods. This directly resolved the effects of SCP_2 on sterol flux between donor and acceptor vesicles. As in the absence of SCP_2, the data displayed biexponential kinetics that could be best described by three sterol fractions (domains) and corresponding exchange half-times for two of them.

In POPC/sterol vesicles, SCP_2 enhanced sterol-exchange kinetics by significantly decreasing the $^1t_{1/2}$ from 27 to 11 min and $^2t_{1/2}$ from 145 to 93 min (Table 1). Concomitantly, SCP_2 increased the size of the rapidly exchangeable sterol domain f_1 from 0.11 to 0.15 and the nonexchangeable domain f_3 from 0.21 to 0.37. These domains increased at the expense of f_2 which decreased from 0.70 to 0.48. As compared to PC, inclusion of PE (another net neutral zwitterionic phospholipid) did not significantly alter the pattern of effect of SCP_2 on any of the sterol exchange parameters (Table 1). In contrast, inclusion of 10 mol % anionic phospholipid markedly potentiated the effects of SCP_2 on sterol exchange halftimes. Depending on the specific anionic phospholipid composition (10 mol % PS, PI, CL), SCP_2 decreased the exchange half-time of the rapidly exchangeable fraction (f_1) from 27 in PC vesicles (Table 1) to 1 min or less (Table 2) and decreased the exchange half-time for the second slowly exchangeable fraction (f_2) from 145 min (Table 1) to 21–83 min (Table 2). The rapidly exchangeable sterol fraction, f_1, was increased from 0.11 to 0.48–0.53 (Table 2) at the expense of the slowly exchangeable fraction, f_2. SCP_2 increased the size of the nonexchangeable domain in PS and PI containing SUV. Thus, the net effect of SCP_2 was to dramatically stimulate sterol exchange in all SUV membranes. This effect was due both to enhanced half-times of exchange as well as to conversion of slowly exchangeable sterol domains into rapidly exchangeable sterol domain. The anionic phospholipid potentiation of SCP_2 action was specific since the presence of PE, another neutral zwitterionic phospholipid in POPC/sterol vesicles, did not significantly enhance the SCP_2-mediated sterol changes in half-times or domains over those observed with POPC/sterol vesicles.

The SCP_2-mediated modulation of sterol domains and enhancement of exchange was not a generalized nonspecific effect of sterol binding proteins. Liver fatty acid-binding protein (L-FABP) and SCP_2 both bind dehydroergosterol and/or cholesterol (Fischer et al., 1985; Nemecz & Schroeder, 1991; Schroeder et al., 1990b). Yet, L-FABP was without effect on sterol exchange kinetics and domains in neutral zwitterionic SUV (Table 1). Anionic phospholipid (e.g. PS, PI, or CL) did not induce L-FABP to enhance sterol exchange between SUV. Moreover, when sterol and phospholipid binding to SCP-2 was inhibited by labeling the protein with *N*-ethylmaleimide, the labeled SCP-2 did not enhance sterol transfer between PC/PS SUV (Woodford et al., manuscript in preparation).

Concentration Dependence of SCP_2-Mediated Sterol Exchange. The range of the SCP-2 concentration used was similar to that in cell cytosol, <3 μM (Paulussen et al., 1989). The enhancement of sterol exchange was dependent on SCP_2 concentration in both POPC/sterol vesicles (Figure 1A, curve 4 vs 3) and PS-containing vesicles (Figure 1B, curve 4 vs 3). These curves and additional data over the SCP_2 concentration

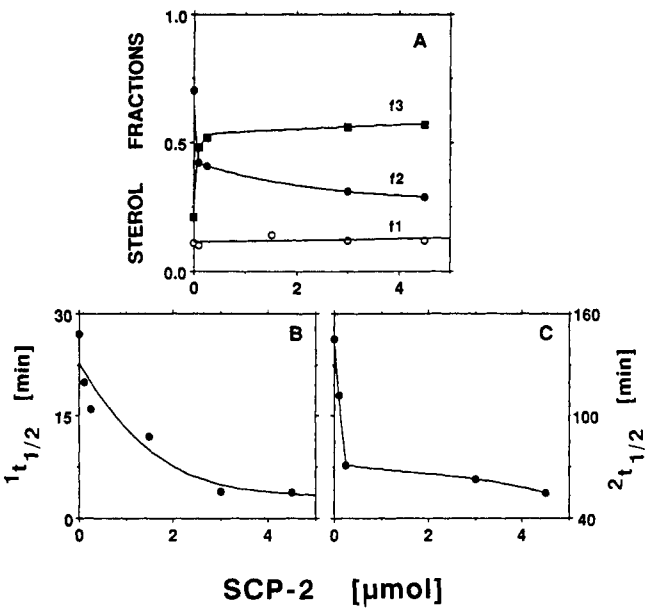

FIGURE 2: Effect of SCP_2 concentration on sterol-exchange kinetics in PC/cholesterol vesicles. Sterol exchange and data processing were performed as described in Materials and Methods and in the legend to Figure 1 in the presence of SCP_2 at indicated micromolar concentrations. Panel A: f_1, rapidly exchangeable fraction in POPC/sterol SUV (65:35); f_2, slowly exchangeable fraction in the same SUV; f_3, nonexchangeable fraction in the same SUV. Panel B: exchange half-time of the rapidly exchangeable fraction. Panel C: exchange halftime of the slowly exchangeable fraction.

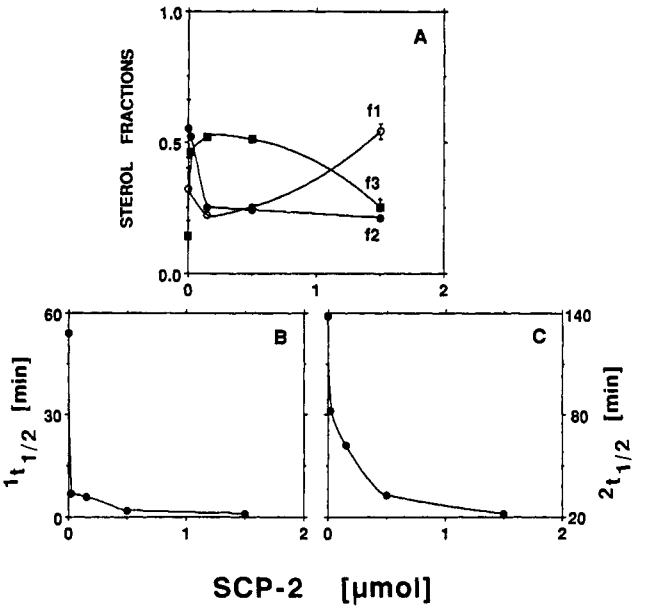

FIGURE 3: Effect of SCP_1 concentration on sterol-exchange kinetics in PS/PC/cholesterol vesicles. Panel A: f_1, rapidly exchangeable fraction in PS/POPC/sterol SUV (10:55:35); f_2, slowly exchangeable fraction in the same SUV; f_3, nonexchangeable fraction in the same SUV. Panel B: exchange half-time of the rapidly exchangeable fraction; Panel C: exchange half-time of the slowly exchangeable fraction.

range 0–5 μM were resolved into individual half-times and domains. The kinetics of sterol exchange were stimulated by SCP_2 in a dose-dependent and saturable manner dependent on vesicle composition. The changes in sterol fractions and exchange half-times in SUV as a function of SCP_2 concentration are shown in the Figures 2 and 3, respectively. There were significant qualitative and quantitative differences between the exchanges involving PC/sterol (Figure 2A-C) and PC/PS/sterol (Figure 3A-C) vesicles.

Qualitatively, increased SCP_2 did not enhance sterol exchange in PC/sterol vesicles by changes in fractions but instead by eliciting a 6-fold decrease in $^1t_{1/2}$ and a 2-fold decrease in $^2t_{1/2}$ (Figure 2). In contrast, increased SCP_2 enhanced sterol exchange in PC/PS/sterol vesicles (1) by increasing f_1 (at the expense of f_2 and, at high concentration of SCP_2, of f_3) and (2) by dramatically decreasing both $^1t_{1/2}$ and $^2t_{1/2}$ (Figure 3).

Quantitatively SCP_2 altered sterol exchange in PC/sterol vesicles differently than in PC/PS/sterol vesicles. Much lower protein concentrations were sufficient for a strong effect on all kinetic parameters in the presence of PS than in pure PC/sterol vesicles. Furthermore, the maximal extent of the SCP_2 effect was usually much higher in PC/PS than PC vesicles (compare Figure 2A-C, Figure 3A-C). This further supported the conclusion that the observed effect of SCP_2 reflected the changes in sterol-exchange kinetics and that the stimulation of SCP_2 activity by acidic phospholipids was mediated by alteration of membrane characteristics sensed by the protein.

The acidic phospholipids could modulate protein binding to the membrane by changes in vesicle surface characteristics. This possibility was tested as follows: the effect of simultaneously altering the number of PS molecules per vesicle as well as SCP_2 concentration was examined. This would be expected to influence the frequency of protein–SUV interactions. In general, the higher the PS content in SUV, the greater was the effect of SCP_2 on sterol domain size, i.e., increased f_1 and decreased f_2 (Figure 4). Likewise, the higher the PS content, the greater was the effect of SCP_2 on corresponding exchange half-times (Figure 5).

Two other observations are noteworthy. The stimulation of sterol exchange was highly significant even at 0.5 μM SCP_2 in vesicles with the lowest PS content (5 mol %). In terms of individual kinetic parameters this concentration of SCP_2 resulted in a 17-fold increase for first fraction f_1 and 4- and 2-fold reduction of $^1t_{1/2}$ and $^2t_{1/2}$, respectively, when compared to corresponding control with no protein. In addition, the stimulatory effect was quantitatively comparable in exchanges including high SCP_2 and low PS or low SCP_2 and high PS. One possible interpretation of these observations is that the overall kinetics was dependent on the number of active SCP_2–SUV complexes which can be varied either by changing SCP_2 availability or number/affinity of binding sites on the membrane by varying the content of acidic phospholipids.

Ionic Interaction between SCP_2 and Membranes: Effect of Charge Screening. The results presented above permit two conclusions. First, the effect of SCP_2 on sterol-exchange kinetics and membrane domains is saturable, and second, direct interaction of the protein with the membrane is probably involved in this effect. SCP_2 is a basic protein with a p*I* near 8.5 (Noland et al., 1980). Since the presence of acidic phospholipids having net negative charge at physiological pH is a prerequisite for maximal stimulation of the SCP_2-mediated sterol exchange, ionic interaction between cationic SCP_2 and anionic vesicle surface could be involved. This possibility was supported by experiments in which the charged groups in SUV and protein were screened as follows.

The dissociation of ionizable groups on the protein and on the membrane surface can be affected by the pH of the assay medium (Table 3). In terms of spontaneous sterol exchange, the half-times of exchange showed only very modest change which were not statistically significant. In contrast, pH significantly modulated SCP_2-mediated sterol exchange. At pH 4.5, the net charge difference between membrane surface and protein is expected to be minimal. With decreasing pH,

Table 3: Effect of pH on Sterol-Exchange Kinetics[a]

pH	SCP-2 (μM)	f_1	f_2	f_3	$^1t_{1/2}$ (min)	$^2t_{1/2}$ (min)
4.5 (4)	0	0.10 ± 0.05	0.81 ± 0.14	0.08 ± 0.03	24 ± 8	128 ± 15
(3)	1.5	0.55 ± 0.07*	0.27 ± 0.12**	0.18 ± 0.06*	3.6 ± 0.3*	31 ± 6**
7.4 (11)	0	0.11 ± 0.04	0.70 ± 0.12	0.21 ± 0.03	27 ± 5	145 ± 14
(3)	1.5	0.15 ± 0.02*	0.48 ± 0.02**	0.37 ± 0.03*	11 ± 2*	93 ± 6**
10.0 (2)	0	0.19 ± 0.02	0.69 ± 0.03	0.12 ± 0.03	32 ± 4	178 ± 25
(3)	1.5	0.55 ± 0.05*	0.20 ± 0.02**	0.25 ± 0.01*	1.2 ± 0.1*	29 ± 5**

[a] SUV composition was 65 mol % POPC:35 mol % sterol (dehydroergosterol in donor, cholesterol in acceptor vesicles). Assay buffers were 10 mM sodium phosphate (pH 4.5), 10 mM PIPES (pH 7.4), 10 mM Tris-HCl (pH 10.0). All other assay conditions and calculations were as in Figure 1 and Table 1.

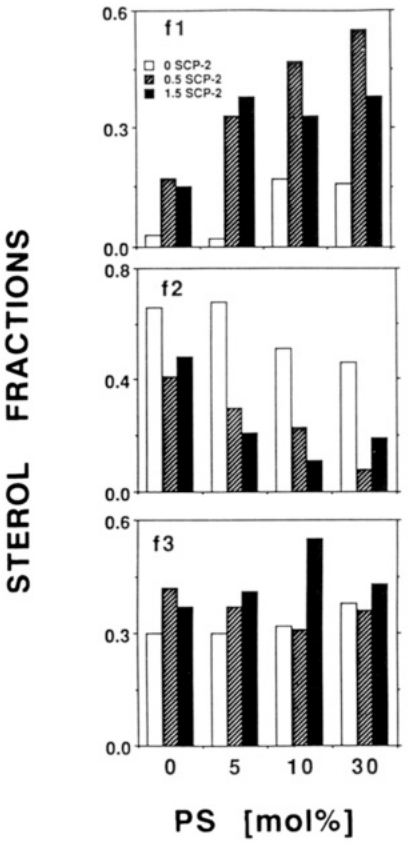

FIGURE 4: Effect of PS concentration on the sterol kinetic fractions in SCP_2-stimulated sterol exchange. Exchange conditions and data processing were as in Materials and Methods and Figure 1. PS content in the SUV was varyed from 0 to 30 mol % with a corresponding decrease in the POPC content from 65 to 35 mol %; sterol content was kept constant at 35 mol %. Symbols with bars represent means ±SEM (n = 3–6): open bars, spontaneous exchanges in the absence of SCP_2; cross hatched bars, exchanges in the presence of 0.5 μM SCP_2; solid bars, exchanges in the presence of 1.5 μM SCP_2.

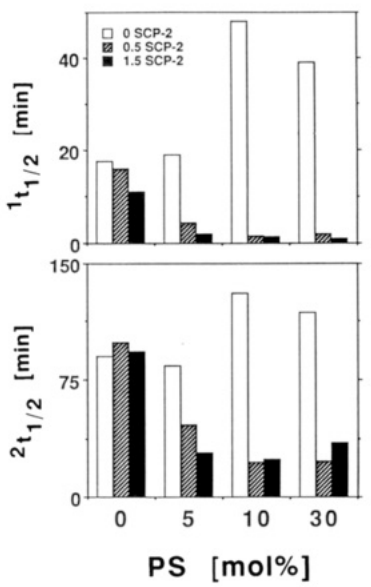

FIGURE 5: Effect of PS concentration on the half-times of SCP_2-stimulated sterol exchange. All conditions and symbols were as in Figure 4.

the SCP_2-mediated fold-enhancement of the first and second half-times of exchange were decreased from 27- and 6-fold (pH 10) to 7- and 4-fold (pH 4.5), respectively. Thus, at low pH, SCP_2 was less effective in stimulating sterol exchange dynamics. This pH effect is understandable if the charge difference between membrane phospholipids and the protein is minimized by protonation of charged residues at low pH, thereby decreasing ionic interactions between the membrane and protein.

Another approach to the analysis of the mechanism of SCP_2 action was the direct screening of membrane surface charges by salts added to the assay medium. Low concentration of salts had small effects on spontaneous sterol exchange: monovalent cations (0.15 M KCl, Table 4) decreased $^1t_{1/2}$ and $^2t_{1/2}$ while f_1 decreased at the expense of f_3. Divalent cations (1 mM $CaCl_2$ or $MgCl_2$, Table 4) had basically similar effects on spontaneous sterol exchange. Monovalent and, to a lesser extent, divalent cations inhibited SCP_2-mediated effects on sterol exchange half-times (Table 4). Likewise monovalent and divalent cations prevented the maximal expression of the SCP_2-medicated effect on fractions f_1 and f_2 (Table 4). This cation inhibition appeared due to both longer exchange half-times, to smaller f_1, and to larger f_3 sterol domains.

The effect of salts on sterol-exchange kinetics was further explored. The polycation neomycin was included in the analysis to avoid the possible effects of osmotic stress that might occur at high concentrations of monovalent and divalent metal ions. As shown above the Tables 1 and 2, SCP_2 increased the size of rapidly exchangeable f_1 and nonexchangeable f_3 sterol domains, while concomitantly decreasing the size of the slowly exchangeable sterol domain f_2. Increasing concentration of neomycin sulfate decreased the SCP_2-mediated increase in sterol domain f_1 size (Figure 6A). However, rather than simply reversing the SCP_2-mediated decrease in f_2 and increase in f_3, neomycin had a little effect on f_2 and instead increased f_3. As described in Tables 1 and 2 above, SCP_2 decreased both of the half-times of sterol exchange, the effect being larger for the rapidly exchangeable $^1t_{1/2}$. Neomycin (Figure 6B,C) reversed the SCP_2-mediated decreases in sterol exchange half-times $^1t_{1/2}$ and $^2t_{1/2}$. Three observations may be made from these results: First, despite the similarity in the effects of neomycin and salts in blocking the effects of SCP_2, there was a striking difference in their effective concentrations, which was 3–4 orders of magnitude higher for KCl than for neomycin. Second, the more positive charges the molecule used for

Table 4: Effect of Ions on SCP_2-Mediated Sterol Exchange

salt	SCP-2 (μM)	f_1	f_2	f_3	$^1t_{1/2}$ (min)	$^2t_{1/2}$ (min)
none (6)	0	0.32 ± 0.08	0.55 ± 0.11	0.14 ± 0.06	54 ± 11	138 ± 32
none (1)	0.5	0.47	0.23	0.31	1.7	22
none (6)	1.5	0.53 ± 0.005	0.20 ± 0.007	0.25 ± 0.01	1 ± 0.1	21 ± 3
KCl (1)	0	0.02	0.59	0.39	17	90
KCl (1)	0.5	0.25	0.34	0.42	12	76
KCl (1)	1.5	0.27	0.32	0.42	7	56
$CaCl_2$ (1)	0	0.17	0.35	0.48	30	114
$CaCl_2$ (1)	1.5	0.20	0.17	0.62	2	28
$MgCl_2$ (1)	0	0.11	0.38	0.52	23	90
$MgCl_2$ (1)	1.5	0.21	0.13	0.63	2.2	33

[a] All assay conditions were as in Figure 1 and Table 1 except for the presence of salts (KCl, $MgCl_2$, and $CaCl_2$) in the assay medium. SUVs were composed of PS/PC sterol (10:55:35). The concentrations of salts were 0.15 M KCl, 1 mM MgCl, and 1 mM $CaCl_2$.

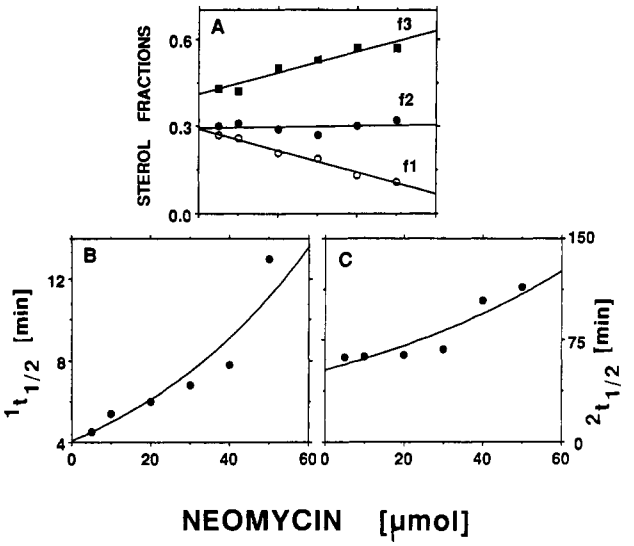

FIGURE 6: Effect of charge screening on the sterol kinetic fractions in SCP_2-stimulated sterol exchange. Exchanges and data processing were performed as specified in Materials and Methods and Figure 1. Exchanges were preformed in the presence of 1.5 μM SCP_2 and neomycin in the specified concentration. SUV were composed of 10 mol % PS, 55 mol % POPC, and 35 mol % sterol: panel A, fractions; panels B and C, half-times of the rapidly and slowly exchangeable fractions, respectively.

screening possessed, the more efficiently it inhibited SCP_2 activity. Third, the effects of high concentrations of KCl were not due to osmotic differences between the outside and inside of the SUV, since neomycin at micromolar concentrations had a similar effect as millimolar concentrations of KCl. Moreover, the inhibition was very similar in exchanges with the salt present both inside and outside of SUV or from the outside only (data not shown).

The results suggested that the binding of cations to the membrane and screening of negative charges on the membrane surface may be the basic mechanism of their inhibitory effect on SCP_2 action. The ionic interaction and subsequent binding of SCP_2 to the membrane appears necessary for the maximal stimulatory effect of this protein on sterol exchange. However, it must be emphasized that the SCP_2-mediated stimulation of sterol-exchange kinetics was not eliminated even at the highest ion concentrations used in this study. The stimulatory effect of the protein on sterol-exchange kinetics was only reduced to the effect seen in the exchanges in essentially electroneutral PC/sterol and PE/PC/sterol SUV.

DISCUSSION

Three major conclusions can be drawn from the results presented here. First, SCP_2 causes major redistribution of sterol molecules among kinetic pools. Second, the overall stimulation of sterol exchange by SCP_2 reflects both shifts in sterol fractions (mainly increase in the rapid exchangeable pool) and significant reduction in the exchange half-times of exchangeable sterol. Third, it appears that ionic interaction between SCP_2 and membrane enhances maximally the stimulatory effect of this protein.

It must be emphasized here that the sterol fractions identified in the present experiments are kinetic and not structural domains, although some relation between them might exist (for review, see Schroeder et al., 1991). These kinetic fractions have to be regarded as dynamic and the changes in their size probably reflect both structural changes (organization of membrane sterols) and kinetic factors (movement of sterol molecules between the domains within the membrane and between the membrane and environment). The membrane curvature is known to affect the size and properties of the sterol domains (Nemecz & Schroeder, 1988). Thus, the difference in sterol-exchange kinetics between PC SUV vs PC/PS SUV may partly be due to the difference in curvature of lipid vesicles resulting from distinct lipid-packing strains caused by different SUV composition. Two-fold increase in the half-time of cholesterol exchange was reported upon substitution of both donor and acceptor SUV for LUV (Fugler et al., 1985). In contrast, in our case the presence of SCP_2 during PC/PS SUV sterol exchange decreased the half-time of the exchange 50-fold as compared to the sterol exchange in the absence of SCP_2 (Table 2). Thus, the effect of distinct lipid packing strains on the SCP_2 enhancement of sterol exchange between SUV resulting from the difference in vesicle curvature is negligible.

The results presented herein showed clearly that ionic interactions between SCP_2 and membrane are necessary for maximal protein activity. This fact seems to be of biological significance as it was shown by van Heusden et al. (1990) that SCP_2 was found in rat tissues both in soluble and membrane-bound form in the cells. Megli et al. (1986) reported that a salt wash of mitochondria removed SCP_2 bound to this fraction and significantly reduced lipid transfer between liposomes and mitochondria. Acidic phospholipids are important for the activity of another protein involved in metabolism of sterols (Caras et al., 1980). However, it must be emphasized that the effect of electrostatic interaction observed in our experiments did not appear to be required for some basal activity. This possibility is supported by the fact that SCP_2 was active in our assays even on electroneutral PC/sterol and PE/PC/sterol vesicles (Table 1). Furthermore, charge screening did not abolish the stimulation of sterol exchange by SCP_2 completely. It was rather reduced to the level found in neutral vesicles. On the other hand, even the "neutral" vesicles have some charges exposed on the surface. Similarly, charge

screening could be only partial in our conditions. Polycationic gentamicin was reported to screen all charges on the membrane surface only at concentrations over 100 μM (Ramsammy & Kaloyanides, 1988). Such high concentrations of polycation neomycin were not used in the present study because they induced major vesicle aggregation. In either case, interaction of the protein with the membrane surface seems to be indispensable for its function and observed effects of SCP_2 on exchange parameters are fairly compatible with such interaction. The nature of the "basal" activity of SCP_2 in mediating sterol exchange between electroneutral vesicles is not known. Perhaps "basal" activity reflects an aqueous transport mechanism independent of the membrane-interaction mechanism.

The interpretation of effects of SCP_2 on sterol-exchange kinetics could be related to mechanisms of the action of this protein. It is generally accepted that the rate of exit of sterol molecules from the membrane into aqueous space is rate limiting in exchange systems with excess of acceptor over donor membranes (see Phillips et al., 1987, for review). The results presented herein as well as the results from other laboratories suggest that direct interaction of SCP_2 with donor membranes plays a major role in this process. Such interaction would cause major changes in membrane sterol-domain structure, exposing a larger number of sterol molecules to an environment favorable for their exit from membrane. The possibility of structural changes induced by protein–membrane interaction is supported by other experimental observations. Several authors reported that binding of cytochrome *c* to lipid monolayers or model bilayers affected their structure and/or permeability (Teissie, 1981; Rietveld et al., 1985; Demel et al., 1989). Ionic interactions between cytochrome *c* and acidic phospholipids in the membrane appeared to be important as was the case with SCP_2. Even more interesting might be the observation of Demel et al. (1989) that apocytochrome *c* was able to mediate the interaction between monolayers and large unilamellar vesicles (LUV) from the subphase. This interaction was accompanied by the transfer of radiolabeled phospholipid from the LUV to monolayers. Almost identical results were obtained in the same laboratory using a similar experimental approach with SCP_2. SCP_2 was shown to penetrate phospholipid monolayers as well as to stimulate the transfer of radiolabeled cholesterol between the monolayer and LUV (Van Amerongen et al., 1989). Redistribution of sterol molecules in kinetic pools in SCP_2-stimulated exchanges among SUV sould be consistent with a similar penetration of protein into the SUV membrane bilayer. Membrane sterol-domain rearrangement induced thereby would result in increased exposure of sterol molecules to aqueous environment due to water penetration into the lipid bilayer as well as to relaxation of specific interactions between sterol molecules and phospholipids in the membrane. Both of these factors could contribute to the enhanced rate of sterol desorption in the presence of SCP_2. The sterol domain corresponding to the rapidly exchangeable domain has been shown to be more aqueous accessible (Nemecz & Schroeder, 1988).

It must be considered that SCP_2 may act as a sterol carrier protein in the aqueous space. Certainly it can bind sterols (Schroeder et al., 1990). Such a mechanism could account for the "basal" SCP_2-mediated sterol-exchange activity. However, sterol binding alone does not explain the effects of anionic phospholipids. Another sterol binding protein, L-FABP (Fischer et al., 1985; Nemecz & Schroeder, 1991), did not significantly enhance sterol transfer between SUV, regardless of the presence of anionic phospholipids (Tables 1 and 2).

In conclusion, SCP_2 appears to enhance sterol exchange between membranes primarily by interaction with the membrane surface. This interaction alters sterol domains and sterol desorption from the donor membrane. SCP_2 acting by an aqueous carrier mechanism appears less significant. Moreover, the ability of SCP_2 to bind sterols is independent of the protein's ability to enhance sterol transfer. Perhaps the sterol binding may be involved in specific targeting or presentation of sterol substrates in enzymatic reactions involving sterol biosynthesis or esterification.

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