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Investigation of the ligand spectrum of human sterol carrier protein 2 using a direct mass spectrometry assay

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Abstract

Sterol carrier protein 2 (SCP2) has been investigated by nearly native electrospray ionisation mass spectrometry in the presence of long chain fatty acyl CoAs (LCFA-CoAs) and carnitine derivatives of equivalent fatty acid chain length (LCFA-carnitines). Four SCP2 constructs were compared to examine the influence of the N-terminal presequence and the C-terminal peroxisomal targeting signal on ligand binding. Removal of N- or C-terminal residues did not influence ligand binding. The observation that LCFA-CoAs are high affinity ligands for SCP2 was confirmed, while LCFA-carnitines were demonstrated for the first time not to interact with SCP2. LCFA-CoAs formed non-covalent complexes with SCP2 of 2:1 and 1:1 stoichiometry, which could be dissociated by elevating the energy of the ions upon entrance to the mass spectrometer. A fluorescence-competition assay using Nile Red butyric acid confirmed the mass spectrometric observations in solution. The physiological significance of the lack of LCFA-carnitine binding by SCP2 is discussed.

Keywords: Sterol carrier protein 2 (SCP2); Non-specific lipid transfer protein; Fatty acyl CoA; Fatty acyl carnitine; Electrospray mass spectrometry; Nile Red butyric acid

Sterol carrier protein 2 (SCP2)[1] bears something of a misnomer—while sterol transfer is within the capacity of SCP2, it has over the years been found to be a non-specific lipid transfer protein (nsLTP) with a broad ligand spectrum [1]. Originally, SCP2 had only been unambiguously identified in higher vertebrates but recently SCP2 sequences have also been found across taxa, including bacteria, archaea, fungi, insects, nematodes and plants [2–5]. In addition to displaying varied tissue distribution and diverse subcellular locations as well as a broad ligand spectrum, SCP2 domains are found in variable molecular contexts [1]. For example mammalian 58 kDa SCPx consists of SCP2 fused C-terminal to 3-oxoacyl-CoA thiolase (reviewed in [1]) while the 80 kDa multi-functional enzyme MFE-2 comprises an N-terminal (3*R*)-hydroxyacyl-CoA dehydrogenase, a 2-enoyl-CoA hydratase 2 domain and a C-terminal SCP2-like domain [6]. Further to this, different isoforms of the same gene product can be found in a single cell—the *SCPx* gene can produce an alternate transcript encoding 15 kDa preSCP2 [1,7], identical to the C-terminal domain of SCPx. Both SCPx and preSCP2 carry a type 1

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[1] *Abbreviations used:* CV, cone voltage; ESI-MS, electrospray ionisation mass spectrometry; GST, glutathione-*S*-transferase; MFE-2, peroxisomal multifunctional enzyme 2; mSCP2, mature peroxisomal form of SCP2; nd, not determined; NR-BA, nile red butyric acid; nsLTP, non-specific lipid transfer protein; preSCP2, translated cytosolic precursor of SCP2; PTS1, peroxisomal targeting signal type 1; SCP2, sterol carrier protein 2; SCPx, 3-oxoacyl-CoA thiolase/SCP2 bifunctional protein; TEV, tobacco etch virus; LCFA, long chain fatty acid.

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peroxisomal targeting signal (PTS1) tripeptide at the extreme C-terminus [8] and both appear to be substrates for intraperoxisomal proteolytic cleavage to yield 13 kDa mature mSCP2 [1,9,10].

Intraperoxisomal function of SCP2 has received considerable attention. Amongst the numerous ligands of SCP2 are LCFAs (discussed extensively in [10,11] with structural analysis in [12,13]) and LCFA-CoAs [14–18] as well as a number of intermediates of LCFA β-oxidation—a primary function of the peroxisome [16]. In addition to the high affinity for these ligands, the observed direct association of peroxisomal SCP2 with β-oxidation enzymes acyl CoA oxidase, thiolase A and MFE-2 [19] has led to the suggestion that a function of SCP2 is to stabilise LCFA β-oxidation intermediates and to present them to the appropriate enzymes for further processing (reviewed in [20]).

Several ligand-binding/transfer assays have been developed as a necessity to address questions regarding the diverse distribution and activity of SCP2 (reviewed in [1]). We have employed a novel and direct *in vitro* assay using "soft" or nearly native electrospray ionisation mass spectrometry (ESI-MS) and verified the results with a competitive binding assay, using a derivative of Nile Red, Nile Red butyric acid (NR-BA, [17]). We have examined LCFA-CoA binding to four variant forms of human SCP2: pre- and mSCP2, each in the presence and absence of the C-terminal PTS1 tripeptide (Fig. 1a). These assays demonstrated tight binding of a set of LCFA-CoAs, concurring with published data [14–17]. However, a set of LCFA-carnitines—a pool of which are present in the peroxisomal matrix [21,22]—were found not to bind to any SCP2 variant.

Materials and methods

Materials

Unless otherwise stated, all chemicals were obtained at the highest available purity from Sigma–Aldrich Chemie GmbH (Munich, Germany). LCFA-CoAs and LCFA-carnitines were supplied at >95% purity by Larodan Fine Chemicals (Malmö, Sweden). Restriction enzymes were purchased from New England Biolabs (Ipswich, MA, USA).

Cloning of SCP2 variants

Four PCR primers allowed generation of four SCP2 constructs: 5′-CAG GTC GAC CAT GGG TTT TCC GGA AGC CGC-3′, encoding the 5′ of preSCP2; 5′-GTA GTC GAC CAT GGG CTC TGC AAG TGA TGG ATT TAA-3′, encoding the 5′ of mSCP2; 5′-CCC TCG CCG GCG TCA GAG CTT AGC GTT GCC TGG-3′, encoding the 3′ of the protein with the three codons for the PTS1 residues (Ala, Lys, Leu) and 5′-CAG TCG CCG GCG TCA GTT GCC TGG CTG AAG CTG AAG-3′, encoding the 3′ lacking the PTS1 codons. Using the appropriate primers pairwise, human fibroblast cDNA as PCR template and *Pfu* DNA polymerase (Stratagene, La Jolla, CA, USA), fragments encoding preSCP2 (residues 1–143), mSCP2 (21–143), preSCP2ΔAKL (1–140) and mSCP2ΔAKL (21–140) were generated (Fig. 1a). Insertion into a modified pET24d vector (Novagen, San Diego, CA, USA), called pETM30 (G. Stier, unpublished) between *Nco*I and *Not*I restriction sites gave clones encoding His_6-GST-TEV-SCP2 fusion proteins. Correct plasmid construction was verified by sequencing.

Expression and purification of SCP2 variants

BL21(DE3) CodonPlus-RIL cells (Stratagene, La Jolla, CA, USA) were transformed with SCP2 encoding plasmids. Single colonies were used to inoculate 100 ml LB medium supplemented with 30 μg/ml chloramphenicol and 30 μg/ml kanamycin, and the culture grown overnight at 30 °C. Ten millilitres of the overnight culture was used to inoculate 1 L LB medium, supplemented 30 μg/ml chloramphenicol and 30 μg/ml kanamycin at 37 °C. At OD_{600nm} ~0.5, cultures induced with 0.5 mM IPTG for 6 h. Cells were harvested by centrifugation, washed with 100 mM potassium phosphate (pH 7.4), 5 mM 2-mercaptoethanol (Buffer A), flash frozen in liquid nitrogen and stored at −20 °C until needed. Cell pellets were thawed and resuspended in Buffer A supplemented with Complete protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany) and 0.05% (w/v) phenylmethylsulfonyl fluoride. Cells were lysed by addition of 0.1 mg/ml lysozyme and incubation at 4 °C with agitation for 15 min, followed by sonication. The lysate was kept on ice and cleared by centrifugation (40,000*g*, 45 min, 4 °C). The cleared lysate was subjected to an initial affinity chromatography step by application to glutathione Sepharose 4B resin (GE Healthcare, Uppsala, Sweden). The column was washed with 5 column volumes of Buffer A and bound proteins eluted with two column volumes of Buffer A supplemented with 20 mM reduced glutathione. TEV cleavage was conducted overnight at 4 °C using 0.5 μg His_6-TEV per 100 μg fusion protein, estimated by $A_{280nm} = 1$ being equivalent to 1 mg fusion protein. An additional 0.5 μg His_6-TEV per 100 μg fusion protein was added and the digest incubated at 30 °C for a further 2 h. The cleavage product was applied to nickel nitrilotriacetic acid agarose resin (Ni–NTA, QIAgen, Hilden, Germany) and the column flow-through retained. Residual (i.e. uncleaved) fusion proteins, His_6-TEV and His_6-GST were efficiently removed by this second affinity chromatography step. The Ni–NTA flow-through was concentrated using 5 kDa nominal molecular weight cut-off ultrafilters (Sartorius AG, Göttingen, Germany) and finally subjected to size exclusion chromatography using a Superdex 75 (16/60 or 10/30) column (GE Healthcare, Uppsala, Sweden), pre-equilibrated in Buffer A. Final protein purity was analysed by SDS–PAGE and mass spectrometry. In all cases, proteins were judged to be >95% homogeneous. Protein concentrations were determined by A_{280nm} using extinction coefficients calculated by the method of Gill and von Hippel [23] and protein denatured in 8 M urea. Pure protein yields per litre cell culture were routinely 40 mg for all forms of SCP2. These purification products are referred to as apo-SCP2 throughout this paper, while holo-SCP2 refers to SCP2 to which ligands have subsequently been added.

Circular dichroism spectropolarimetry

CD spectra were measured on a J-810 spectropolarimeter (Jasco, Easton, MD, USA). SCP2 variant samples were prepared by dialysis (Spectra-Por dialysers, Carl Roth GmbH, Karlsruhe, Germany) against 10 mM potassium phosphate (pH 7.4). Prior to measurement, samples were diluted to 8 μM and used to fill a 1 mm quartz cuvette (Hellma GmbH & Co., KG, Müllheim, Germany). Spectra were measured from 250 to 190 nm at 20 °C, in 0.2 nm steps with 1 s integration time. Five spectra were accumulated and averaged. Experiments were repeated in the presence of 80 μM stearoyl CoA added from a 2 mM stock solution in 100 mM potassium phosphate (pH 7.0). Buffer and stearoyl CoA background spectra were subtracted. Data were scaled to molar ellipticity (in deg cm^2 $dmol^{-1}$). Secondary structure content was estimated using K2D [24].

Electrospray ionisation mass spectrometry

SCP2 variants were mixed with a threefold molar excess of putative ligands (palmitoyl CoA, stearoyl CoA, oleoyl CoA, acetyl CoA, palmitoyl carnitine, stearoyl carnitine and oleoyl carnitine) (Fig. 1b) from 2 mM stocks in 100 mM potassium phosphate (pH 7.0). A 100-fold molar excess was used for acetyl CoA and the three LCFA-carnitines from 100 mM stocks in methanol. Samples were subjected to exhaustive washing with

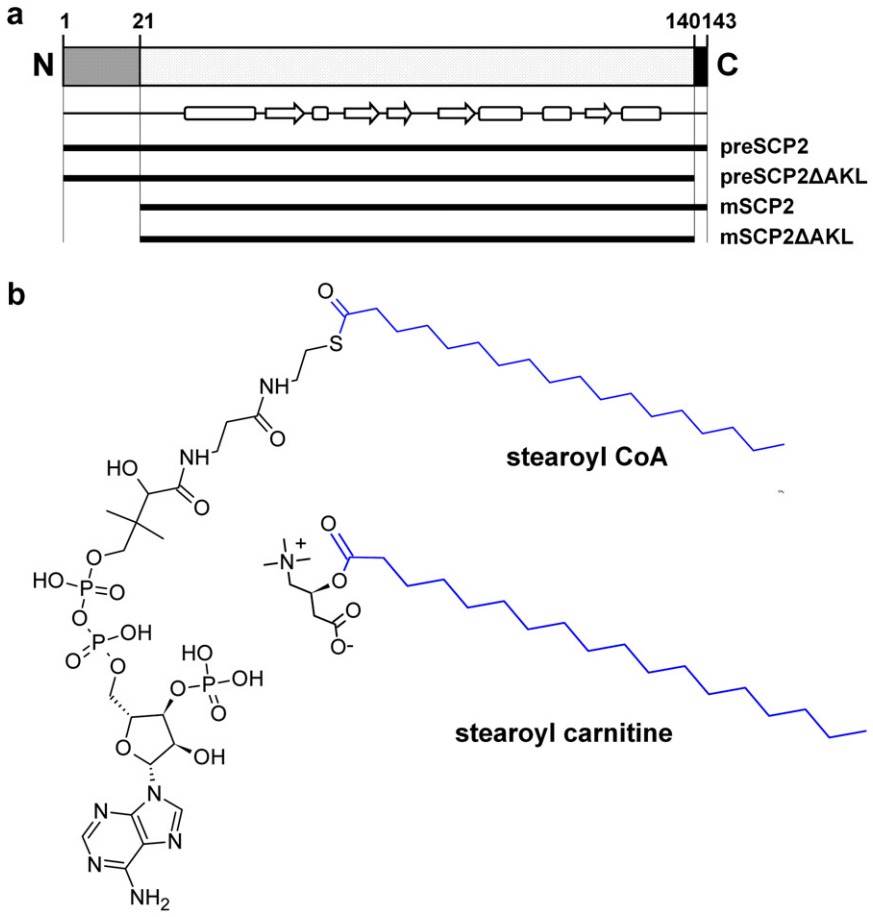

Fig. 1. SCP2 organisation and putative ligands. (a) The top bar shows the overall organisation of SCP2—the main lipid-binding core is shaded in light grey, the 20 residue N-terminal presequence in dark grey and the C-terminal PTS1 tripeptide in black. Approximate positions of secondary structures in rabbit mSCP2 [26] are indicated, with helices shown as rectangles and β-strands as arrows. The four SCP2 variants used in this study are indicated below. (b) Chemical structure comparison of stearoyl CoA and stearoyl carnitine. Aliphatic chains are shown in blue and head groups in black. In addition to the stearoyl derivatives (C18:0), oleoyl (*cis*-Δ^9 C18:1), palmitoyl (C16:0) and acetyl (C2:0) derivatives have been used.

200 mM ammonium acetate (pH 7.4) using 5 kDa nominal molecular weight cut-off ultrafilters (Sartorius AG, Göttingen, Germany) and concentrated to approximately 5–20 μM. Mass spectra were recorded on a Micromass LCT instrument (Waters, Elstree, UK) operating in positive ion mode. The potential between the electrospray needle and the sample cone was generally set around 1500 V and the cone voltage (CV) was varied (between 40 and 200 V) to analyse the dissociation of the complexes. Electrospray needles were prepared as described [25]. Data were collected and analysed using MassLynx 3.5 (Waters, Elstree, UK). Percentage holo-SCP2 at a given CV was defined as [(total area of holo-SCP2 charge states)/(total area of holo- and apo-SCP2 charge states)] × 100.

Nile Red butyric acid fluorescence-competition assay

Nile Red butyric acid (NR-BA) was synthesized by Oliver Wichmann, EMBL-Heidelberg, Germany. The synthesis, fluorescent properties and use in ligand competition assays of NR-BA are described in [17]. In brief, samples of SCP2 variants were prepared at 8 μM in 100 mM potassium phosphate (pH 7.0) and preincubated with 250 nM NR-BA for 30 min. Fluorescence emission spectra were recorded at 20 °C on a QuantaMaster C-61/2000SE spectrofluorimeter (Photon Technology International, Birmingham, NJ, USA) in a 50 μl quartz cuvette (Hellma GmbH & Co., KG, Müllheim, Germany). An excitation wavelength of 540 nm and an emission scan of 570–700 nm, with step size of 3 nm and 1 s integration time were used. SCP2 samples were titrated with aliquots of LCFA-CoAs from a 2 mM stock solution in 100 mM potassium phosphate (pH 7.0). Comparison titrations were made using LCFA-carnitines, titrated from a 100 mM stock in methanol, thus at the maximal concentration of LCFA-carnitine used during titrations (846 μM) methanol was present at ~0.8% (v/v). Where low concentrations of LCFA carnitines were required, the 100 mM stock was diluted to 2 mM in 100 mM potassium phosphate (pH 7.0). Data were corrected for dilution effects during titration and background spectra subtracted. Standard deviations were taken from three replicate experiments.

Results

CD analysis of SCP2

Four variants of human SCP2 have been prepared recombinantly—the wild-type translation product preSCP2 and the mature, intraperoxisomal form, mSCP2 [1] in addition to mutants of each lacking the three C-terminal PTS1 residues (Ala141-Lys142-Leu143), preSCP2ΔAKL and mSCP2ΔAKL. Far-UV CD demonstrated that these variants are correctly folded. Fig. 2a shows spectra from each variant prepared in the holo-state by saturation with the high-affinity ligand stearoyl CoA [14,16]. Spectra of preSCP2 and preSCP2ΔAKL are nearly identical to each

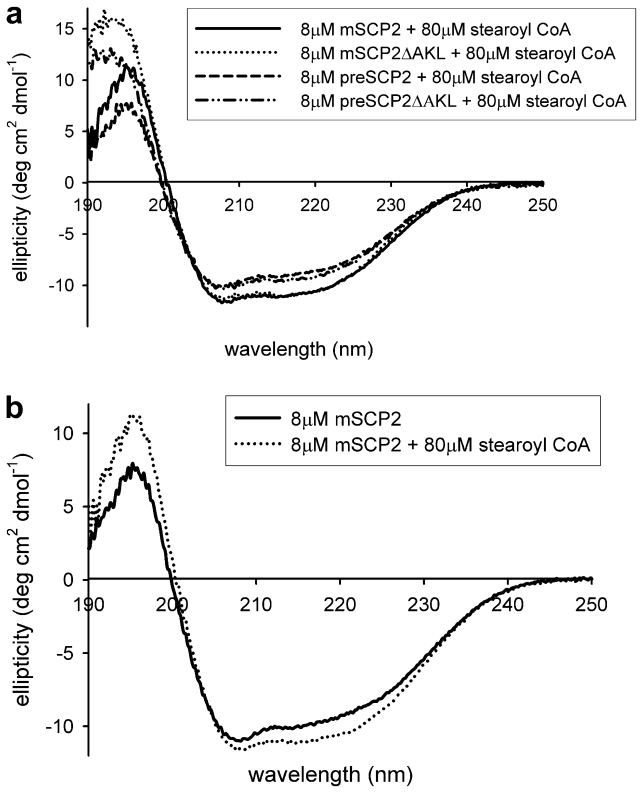

Fig. 2. CD analysis of SCP2. (a) Far-UV CD spectra of the four isoforms of SCP2. Holo-SCP2 was prepared by saturation with stearoyl CoA. (b) Far-UV CD spectral comparison of apo- and holo-mSCP2. Spectra were measured at 20 °C with samples in 10 mM potassium phosphate (pH 7.4).

other, as are mSCP2 and mSCP2ΔAKL spectra, with the spectral quality deteriorating below about 200 nm. The preSCP2/preSCP2ΔAKL spectra show slightly weaker maximum and minima compared to those of mSCP2/mSCP2ΔAKL, accounted for by a lower regular secondary structure content: the software K2D [24] estimates preSCP2 and preSCP2ΔAKL to have approximately 31% α-helical content and 22% β-sheet, while mSCP2 and mSCP2ΔAKL carry approximately 33% α-helix and 22% β-sheet, the sharp minimum at 208 nm indicative of increased percentage helical content. These values are in good agreement with the secondary structure found in the crystal structure of rabbit mSCP2 ([26], Fig. 1a), with 36.6% α-helix and 26.6% β-sheet content as evaluated by PROMOTIF [27]. CD comparison of apo-SCP2 and holo-SCP2 reveal a small increase in ordered secondary structure upon addition of stearoyl CoA in the case of all four isoforms. Fig. 2b shows holo- and apo-mSCP2 spectra as an example.

ESI-MS analysis of ligand binding to SCP2

So-called "soft", or nearly native, electrospray ionisation mass spectrometry (reviewed in [28]) has been used to conduct a direct analysis of ligand binding by SCP2. Complexes of LCFA-CoAs (stearoyl, palmitoyl and oleoyl) could be identified with each of the four SCP2 variants used. Each of these complexes behaved in a similar manner. Fig. 3 shows representative data in which oleoyl CoA was used as ligand. Fig. 3 focuses on major *m/z* peaks found for SCP2 complexes with charge 7+ and 8+. Apo-preSCP2 was measured to have a mass of 15531.4 Da, which is close to the calculated mass of 15528.9 Da; preSCP2ΔAKL was measured at 15219.8 Da (15216.4 Da calculated); mSCP2 at 13472.5 Da (13471.6 Da calculated) and mSCP2ΔAKL at 13161.1 Da (13159.2 Da calculated). In each preparation, in addition to apo-SCP2, two holo-SCP2 states could be found with mean masses 1032.3 and 2064.6 Da over apo-SCP2, consistent with the presence of one or two molecules of oleoyl CoA in complex with SCP2 (Fig. 3). Dissociation of complexes of SCP2 with stearoyl CoA could be induced by increasing the instrumental CV. Fig. 4 shows the dissociation of preSCP2-stearoyl CoA as CV is gradually increased from 80 to 140 V. The intensities of apo-preSCP2 peaks increase relative to those representing holo-SCP2, demonstrating that non-covalent complexes are present. Each of the four SCP2 variants were subjected to the same dissociation from stearoyl CoA. At CV = 80 V, holo-SCP2, regardless of whether it assembled into 1:1 or 1:2 complexes, is present as approximately 50–55% of total SCP2, decreasing to approximately 10% at CV = 150 V. The rate of decrease, presenting the rate of dissociation, is found to be similar for each SCP2 variant (Fig. 5). In addition to the three LCFA-CoAs, four ligands were tested which showed no interaction with any SCP2 variant under these assay conditions—acetyl CoA, stearoyl carnitine (Fig. 6), palmitoyl carnitine and oleoyl carnitine. In every case mass spectra were measured which were indistinguishable from those of apo-SCP2, even at low CV.

Competitive displacement of NR-BA from the ligand binding pocket of SCP2 by physiological ligands

To verify the data obtained from the ESI-MS assay in an aqueous environment an indirect assay was employed, utilizing NR-BA as an environmentally sensitive competitive ligand for SCP2 [17]. Exposure of SCP2, preincubated with 250 nM NR-BA, to excitation at 540 nm produces a strong fluorescence maximum at 618 nm. Titration with palmitoyl CoA gives rise to a dose-dependent decrease in fluorescence intensity concomitant with a red-shift to 657 nm, which at the titration end point has a relative fluorescence intensity of about 26% compared to the absence of palmitoyl CoA (Fig. 7a). The EC_{50} with 8 μM mSCP2 was found at 8.8 μM palmitoyl CoA, a molar ratio of 1:1.10. For all other SCP2 variants and LCFA-CoAs tested, EC_{50} values of a similar magnitude (7.6–8.8 μM) were found (Table 1). In contrast, acetyl CoA and the LCFA carnitines tested did not give rise to a red-shift and thus are apparently unable to displace NR-BA, even at more than 100-fold molar excess over SCP2. Representative data are shown for mSCP2 titrated with palmitoyl carnitine in Fig. 7b.

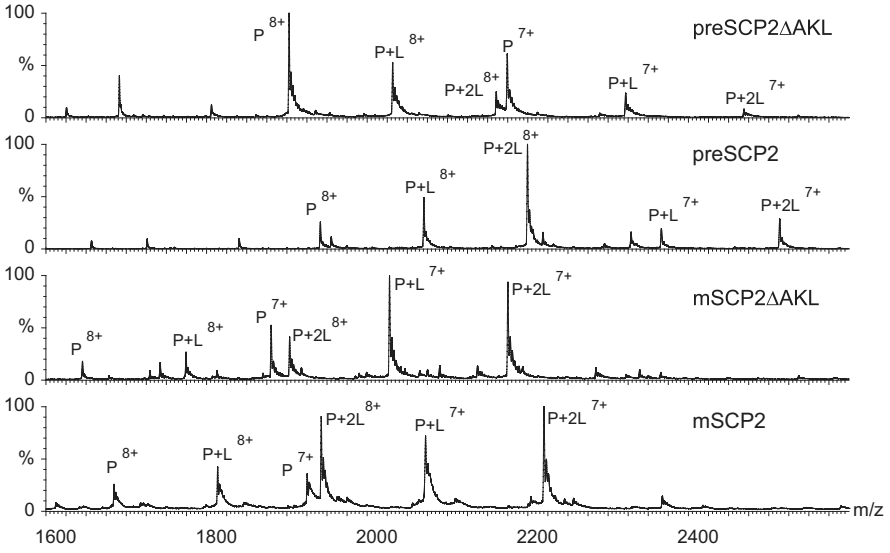

Fig. 3. ESI-MS analysis of four of SCP2 isoforms in the presence of oleoyl CoA. The 7+ and 8+ charge species are labelled. Unliganded SCP2 protein charged species are labelled P. Mass signatures indicative of the presence of a 1:1 complex of SCP2 with one oleoyl CoA ligand are marked with P+L; 1:2 complexes with P+2L. CV was set at 110 V.

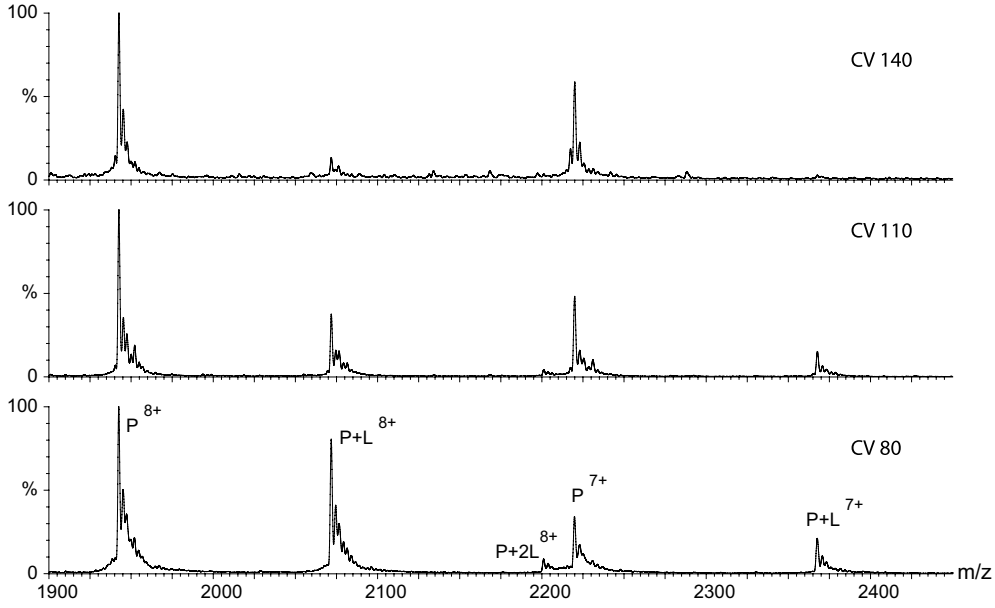

Fig. 4. Dissociation of the preSCP2:stearoyl CoA complex as a function of cone voltage. Charged species are labelled as in Fig. 3. Note the increase in relative abundance of apo-preSCP2 (P peaks) with increasing CV.

Discussion

In this study we have used four variants of human SCP2, with or without the N-terminal presequence and the C-terminal PTS1 motif, in a direct analysis of lipid binding. Each of the four variants, regardless of the presence or absence of either presequence or PTS1, were found by far-UV CD to be natively folded, as estimated by far-UV CD and confirming previous structural data [3,13,26,29–31]. Previous spectroscopic studies [32,33] have shown that the presequence of SCP2 does not display regular secondary structure—a conclusion confirmed by the CD data presented here. In contrast to one earlier CD study [34], in which the presence of the presequence was found to significantly reduce the ordered secondary structure content of SCP2, our data are consistent with available NMR data [18,31,32] indicating that the overall fold of preSCP2 and mSCP2, aside from the coiled presequence, does not change. We additionally demonstrate that removal of the PTS1 tripeptide does not alter SCP2 secondary structure and thus is not critical for SCP2 folding, as could be expected from high-resolution structural analyses

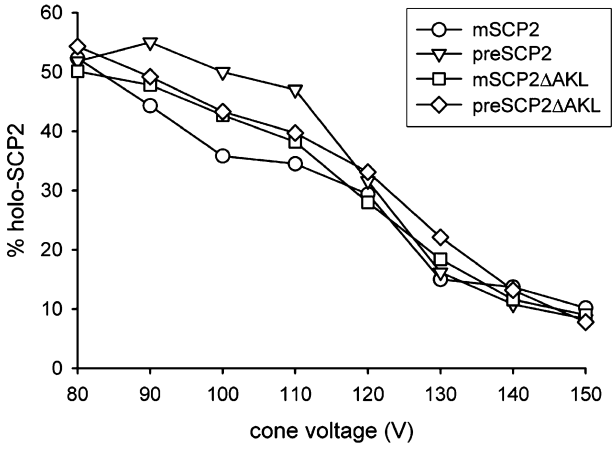

Fig. 5. Comparison of the relative partition of four holo-SCP2 variants as a function of cone voltage. Data are shown for SCP2:stearoyl CoA complexes. Holo-SCP2 is given as a percentage of total (i.e. holo- and apo-) SCP2.

[26,30,31] in which the C-terminal segment of SCP2 is found to be in a coiled conformation. Crystal structures of liganded SCP2 [13,29] show the C-terminal segment adopting an α-helical structure "capping" the ligand binding pocket. This coil-helix transition upon ligand binding may to some degree explain the "tightening" of secondary structure we observe when SCP2 is loaded with stearoyl CoA.

"Soft" ESI-MS has frequently been used to study biomolecular complexes in a nearly native state in the gas phase, for example protein/protein [35], enzyme/cofactor [36] and protein/lipid [25,37]. We have adapted this kind of assay to analyse SCP2/lipid. We have thus determined that qualitatively these four SCP2 variants interact comparably with a set of ligands. High affinity LCFA-CoA ligands can be found in 1:1 and 2:1 stoichiometry with SCP2. It has previously been reported that SCP2 has a capacity to bind two LCFA-CoA ligands [10,17], but not more than one cholesterol molecule [10]. These earlier reports have been suggestive of a sequential loading of SCP2, first with one LCFA-CoA ligand and then with the second. The ESI-MS assay applied in this study allows direct visualisation of apo- and holo-SCP2 in the presence of one and two LCFA-CoA ligands. The presence of three molecular species (apo-SCP2, 1:1 and 2:1 holo-SCP2) in the same mass spectrum, however, does not allow determination of the sequential nature of ligand binding or dissociation. While dissociation of total holo-SCP2 to apo-SCP2 can be followed by manipulation of cone voltage, revealing similar dissociation behaviour for each SCP2 variant, we cannot detect a regular pattern of dissociation from 2:1 to 1:1 to apo-SCP2 from these spectra as the relative abundance of 2:1 and 1:1 complexes seems to be strongly variable. However, our data show that the overall ratio of holo-SCP2 to apo-SCP2 responds roughly linearly to variation in CV. Thus it could be postulated that the dissociation occurs by a single step but presently we cannot speculate on the exact mechanism.

During the early stages of assay optimisation, we have examined whether the presence of methanol may influence ligand binding to SCP2. Use of methanol in the assay could expand the repertoire of ligands that may be tested in this assay by increasing the solubility of lipid compounds under test. While a previous *in vitro* study showed that ethanol inhibits binding of some fatty acid derivatives to SCP2 [38], in this mass spectrometric assay we did not detect and inhibition of stearoyl CoA binding to SCP2 as a result of addition of methanol up to 25% (v/v). Indeed, although

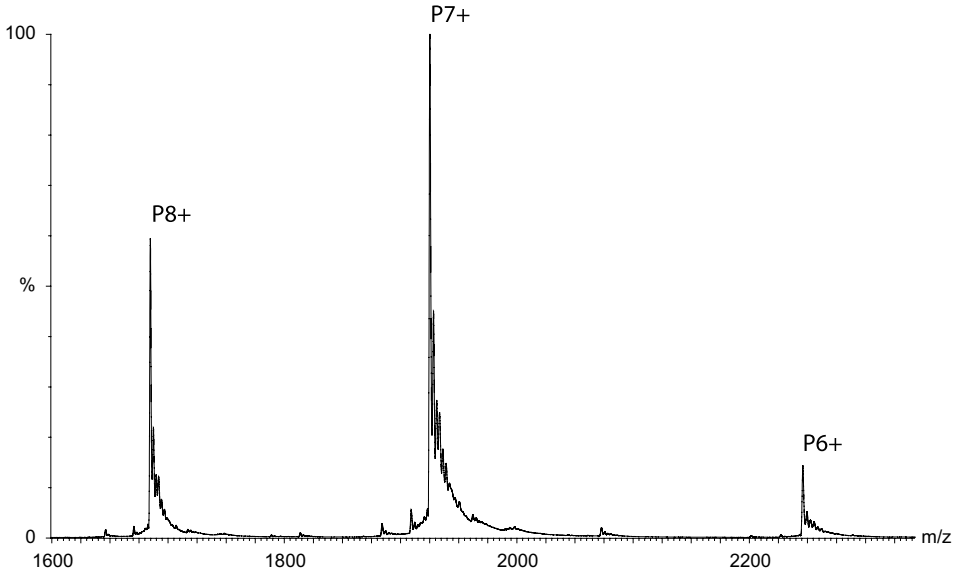

Fig. 6. Mass spectrum showing mSCP2 preincubated with stearoyl carnitine. Measured at CV = 80 V (at which stearoyl CoA interacts well with SCP2), *m/z* peaks , can only be seen for the free protein (P +6, +7 and +8 species) and no complexes with stearoyl carnitine. The absence of complexes was found with all four SCP2 variants and with palmitoyl carnitine, oleoyl carnitine and acetyl CoA.

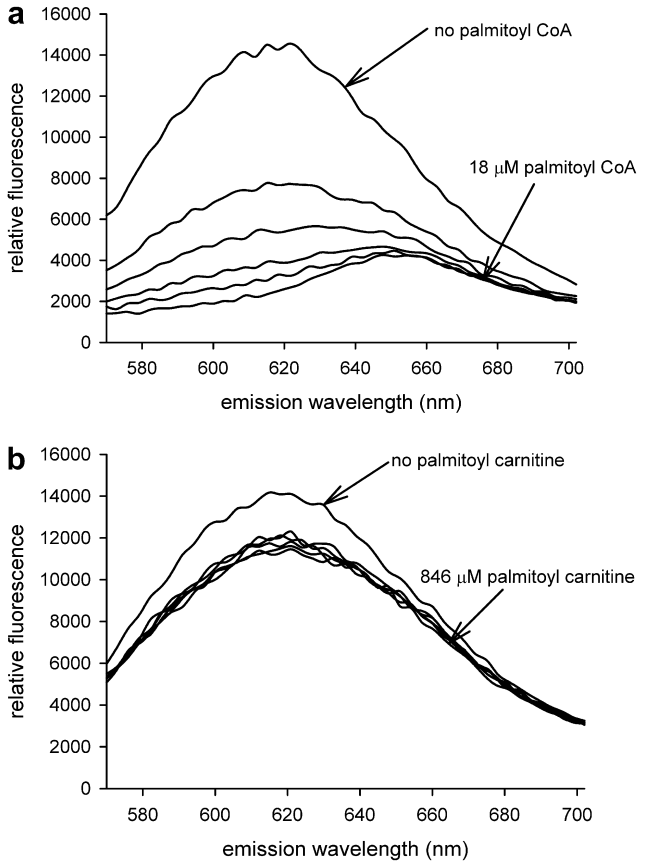

Fig. 7. Nile Red butyric acid analysis of ligand binding to SCP2. (a) Competitive displacement of 250 nM NR-BA from mSCP2 by titration with palmitoyl CoA. (b) Titration with 100-fold higher concentrations of palmitoyl carnitine does not result in NR-BA displacement. Emission scans were carried out on samples of mSCP2 (8 μM) in 100 mM potassium phosphate (pH 7.0) at 20 °C, with an excitation wavelength of 540 nm.

not strictly quantitated, the presence of methanol seemed to enhance stearoyl CoA binding, maybe as a result of improved ligand solubility or more efficient electrospray ionisation (data not shown). We could not detect any complexes of SCP2 with methanol in our spectra (data not shown) and thus, if methanol directly interacts with SCP2 in must do so only weakly.

SCP2 failed to bind to either acetyl CoA or a set of LCFA-carnitines in the soft-ESI-MS assay. Acetyl CoA has previously been observed to lack the ability to displace *cis*-parinaric acid from SCP2 [14] and has thus provided a convenient negative control ligand in this study. In our assays, no complex was observed with any SCP2 variant after incubation with threefold or even 100-fold molar excess of the acetyl CoA. Therefore, the binding determinant appears to be the long fatty acid chain of the compound and no independent CoA binding site is present. In contrast, none of the tested LCFA-carnitines were found in complex with SCP2, even though they all possess a long fatty acid chain. Therefore, we hypothesize that the carnitine head group could be the determinant in inhibition of binding. While CoA is a polar group, carnitine carries a positive and a negative charge (Fig. 1b). In the available crystal structure of mosquito SCP2 in complex with palmitic acid [13], one carboxylate oxygen of palmitic acid is buried within SCP2 (Fig. 8a)—hydrogen bonding directly with three residues: Arg24, Gln25 and Val26. The other carboxylate oxygen, however, is solvent exposed, hydrogen bonding with Arg15 and Asp20 *via* water molecules [13]. It could be expected that bound LCFA-CoAs will have the head group-fatty acid thioester linkage at this position. Examination of the local environment of this solvent exposed oxygen reveals a high density of charged residues (Fig. 8a). A structure based sequence alignment of rabbit apo-SCP2 (which differs from the human SCP2 used in this study by only three residues) and palmitic acid loaded mosquito SCP2 (Fig. 8b) indicates a helix to coil rearrangement upon ligand binding (residues 41–51 in rabbit SCP2) in the region in which the carboxylate group of palmitic acid binds. Residues in this region within 6 Å (roughly the dimension of a carnitine head group) of the solvent exposed carboxylate oxygen of palmitic acid are well conserved, especially with respect to charge distribution (Fig. 8b). We therefore hypothesize that the charged residue distribution in this carboxylate binding region gives rise to the selectivity for binding of LCFA-CoAs but not LCFA carnitines, the carnitine derivatives being excluded by charge repulsions.

A fluorescence competition assay utilising NR-BA [17] has been applied to verify the results of the soft mass spectrometry assay. NR-BA is of similar size and shape to cholesterol and binds SCP2 in a pocket continuous with the LCFA-CoA binding site [17,18]. The NR-BA assay was previously used to demonstrate the 2:1 binding of linoleoyl

Table 1
EC_{50} values found for SCP2 ligands using the NR-BA competition assay

Ligand	mSCP2	mSCP2ΔAKL	preSCP2	preSCP2ΔAKL
Palmitoyl CoA	8.8 ± 0.9[a,b] (1.10)	7.6 ± 0.7 (0.95)	8.6 ± 0.9 (1.08)	8.2 ± 0.8 (1.03)
Stearoyl CoA	8.6 ± 1.0 (1.08)	8.4 ± 0.7 (1.05)	8.8 ± 1.2 (1.10)	8.8 ± 1.4 (1.10)
Oleoyl CoA	8.2 ± 1.0 (1.03)	8.2 ± 0.9 (1.03)	8.4 ± 0.8 (1.05)	8.4 ± 1.2 (1.05)
Linoleoyl CoA[c]	10.6 ± 0.4 (1.33)	nd	nd	nd

nd, not determined.
[a] Micromolar effective concentration at 50% maximal ligand binding to 8 μM SCP2 sample (EC_{50}). EC_{50} as a ratio of SCP2 concentration is given in parentheses.
[b] Standard deviations are derived from three consecutive measurements.
[c] Based on data from [17].

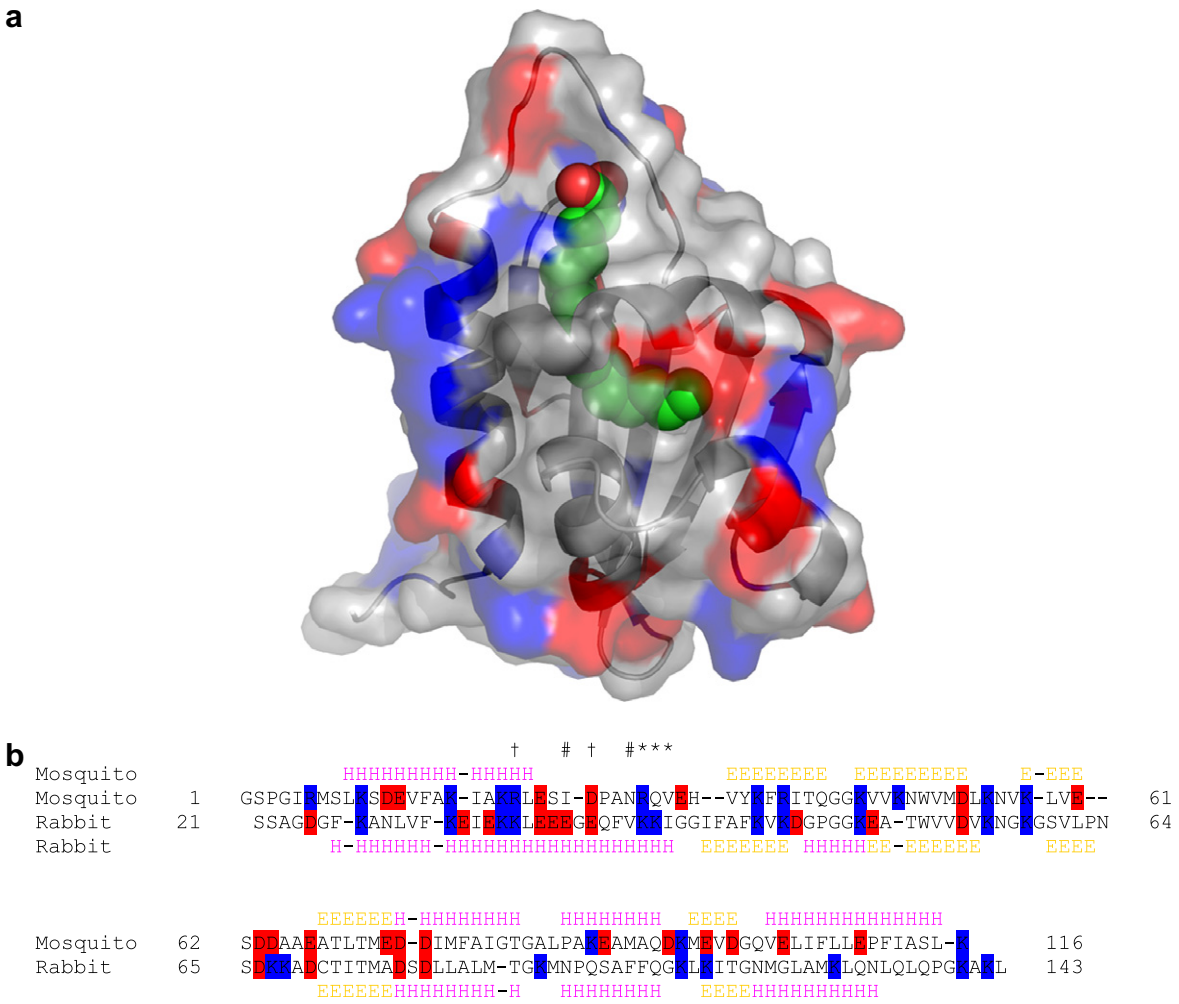

Fig. 8. Structural features of SCP2. (a) The crystal structure of mosquito SCP2 [13]. Basic residues (Arg and Lys) are coloured blue and acidic residues (Asp and Glu) are coloured red. The palmitic acid ligand is shown with atomic spheres, the aliphatic chain in green and carboxylate oxygens in red. Secondary structure cartoons are overlaid with the molecular surface. "N" indicates the N-terminus of the protein to which the presequence of mammalian preSCP2 attaches. (b) Structure based sequence alignment of mosquito SCP2 [13] and rabbit mSCP2 [26]. Sequences were aligned by eye with respect to secondary structure elements and charged residues. Helices are indicated with pink "H" while β-strands are indicated with yellow "E". Basic residues are highlighted in blue and acidic residues in red. Residues lying within 6 Å of the solvent exposed carboxylate oxygen of palmitic acid bound to mosquito SCP2 [26] are shown with "#" of which those labelled with "+" hydrogen bond to this oxygen *via* water molecules. Also within 6 Å of the solvent exposed carboxylate oxygen are three residues which directly hydrogen bond to the buried carboxylate oxygen, labelled with "*". Rabbit SCP2 (shown here) differs from human SCP2 (used in this study) by only three residues—Gly4Ser, Leu80Met and Lys120Asn.

CoA to mSCP2 in a high-fidelity quantitative mode. Here the assay has been applied in a more rapid ligand screening mode. The assay indeed verifies the main conclusions of the ESI-MS experiments—while LCFA-CoA titrations readily displaced NR-BA from SCP2, none of the LCFA-carnitines nor acetyl CoA were able to do so, even when introduced at high concentrations. The EC_{50} values derived from the titration data are consistent with >1 LCFA-CoA ligand binding per SCP2. Comparison of EC_{50} values with that found for linoleoyl CoA [17] show a similar order of magnitude, indicating that the more rapid application of the assay in this study does not significantly compromise efficacy. Taken together with the ESI-MS data, it is clear that neither the presequence nor the PTS1 influences the ligand binding properties of SCP2—indeed, comparison of ligand binding to pre- and mSCP2 has previously demonstrated similar binding constants [16,34] between the isoforms. The presequence and PTS1 are thus implicated in regulating SCP2 localization [9,34].

The physiological relevance of the lack of LCFA-carnitine binding capacity of SCP2 may relate to the dual role of the peroxisome and the mitochondrion in fatty acid β-oxidation. Peroxisomes take up LCFA-CoAs for several rounds of β-oxidation to produce chain shortened carnitine derivatives, which are used both as export substrates from the peroxisome to the cytoplasm and import substrates to the mitochondrion from the cytoplasm (reviewed in [22]). Thus a role for SCP2 can be envisaged in which it assists in retaining the pool of intraperoxisomal LCFA-CoAs for chain shortening but does not inhibit export of

carnitine derivatives which may be transported to the mitochondrion for complete oxidation. Such a role fits well with the previous suggestion that SCP2 functions essentially as a helper molecule in peroxisomal fatty acid oxidation, by it's direct association with both fatty acid oxidation enzymes and various metabolic intermediates [14–18]. By retention, stabilisation and presentation of fatty acid oxidation intermediates suitable for catabolism in the peroxisome, coupled to rejection of carnitine derivatives, SCP2 could significantly increase the efficiency of cellular fatty acid oxidation. One *in vivo* study has indicated that there is a pool of preSCP2 in the cytosol [9]. In this study we have not been able to distinguish any significant difference in activity of preSCP2 or mSCP2 towards fatty acyl carnitines *in vitro*, thus there is no basis for suspecting that the presence of a cytosolic concentration of preSCP2 will interfere with the uptake of fatty acyl carnitine derivatives by the mitochondrion. However, to our knowledge, *in vivo* studies examining the role of SCP2 in regulating the cellular levels of fatty acyl carnitines are lacking and may prove fruitful as a future research objective.

In this present study, we have limited the ligand spectrum examined to only two kinds of fatty acid derivatives, of rather similar fatty acid chains. SCP2 is known to interact with a highly diverse set of ligands [1] and to display preference of some fatty acid derivatives over others [1,14–16]. We propose that the assays employed in this study could be useful in examination of a much wider set of putative SCP2 ligands, especially as the mass spectrometric assay is tolerant to addition of methanol which may be used to enhance ligand solubility. A thorough and systematic analysis of fatty acid derivatives of different chain lengths and degrees of unsaturation, for example, could be readily achieved.

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