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Supernatant Protein Factor in Complex with RRR-α-Tocopherylquinone: A Link Between Oxidized Vitamin E and Cholesterol Biosynthesis

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The vast majority of monomeric lipid transport in nature is performed by lipid-specific protein carriers. This class of proteins can enclose cognate lipid molecules in a hydrophobic cavity and transport them across the aqueous environment. Supernatant protein factor (SPF) is an enigmatic representative of monomeric lipid transporters belonging to the SEC14 family. SPF stimulates squalene epoxidation, a downstream step of the cholesterol biosynthetic pathway, by an unknown mechanism.

Here, we present the three-dimensional crystal structure of human SPF in complex with RRR-α-tocopherylquinone, the major physiological oxidation product of RRR-α-tocopherol, at a resolution of 1.95 Å. The structure of the complex reveals how SPF sequesters RRR-α-tocopherylquinone (RRR-α-TQ) in its protein body and permits a comparison with the recently solved structure of human α-tocopherol transfer protein (α-TTP) in complex with RRR-α-tocopherol. Recent findings have shown that RRR-α-TQ is reduced *in vivo* to RRR-α-TQH_2, the latter has been suggested to protect low-density lipoprotein (LDL) particles from oxidation. Hence, the antioxidant function of the redox couple RRR-α-TQ/RRR-α-TQH_2 in blocking LDL oxidation may reduce cellular cholesterol uptake and thus explain how SPF upregulates cholesterol synthesis.

Keywords: crystal structure; lipid transfer; squalene; α-tocopherylquinone; SEC14-like

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Eukaryotic cells contain multiple membrane compartments with specialized lipid compositions. Assembly of the correct mixture of lipids requires a complex process involving the tight spatial regulation of metabolite biosynthesis and a subsequent step of selective distribution of products between cellular compartments. Members of the eukaryotic SEC14-like family of proteins have emerged as critical components of monomeric lipid transport fulfilling the latter task.[1] Typically, these proteins contain a structural element designated CRAL_TRIO that enables them to shuttle between a soluble and a membrane-associated state in order to pick up or to release their lipid cargo.[2]

Supernatant protein factor (SPF) is an accessory protein for the squalene monooxygenase reaction, the first oxidative step in cholesterol biosynthesis.[3] Sequence analysis classifies SPF as an SEC14-like protein family member.[4,5] Its N-terminal 246 amino acid residues harbor a characteristic CRAL_TRIO lipid-binding motif that defines the family of SEC14-like lipid transfer proteins including phosphatidylinositol/phosphatidylcholine transfer protein (SEC14) from *Saccharomyces cerevisiae*, cellular retinaldehyde-binding protein (CRALBP) and α-tocopherol transfer protein (α-TTP).[6] Although SPF has been reported to facilitate squalene transfer into and between membranes, numerous attempts to show binding of the protein with squalene have been unsuccessful.[7–9] In addition, the identification of

Abbreviations used: SPF, supernatant protein factor; α-TTP, α-tocopherol transfer protein; RRR-α-T, RRR-α-tocopherol; RRR-α-TQ, RRR-α-tocopherylquinone; LDL, low-density lipoprotein; TAP, tocopherol-associated protein.

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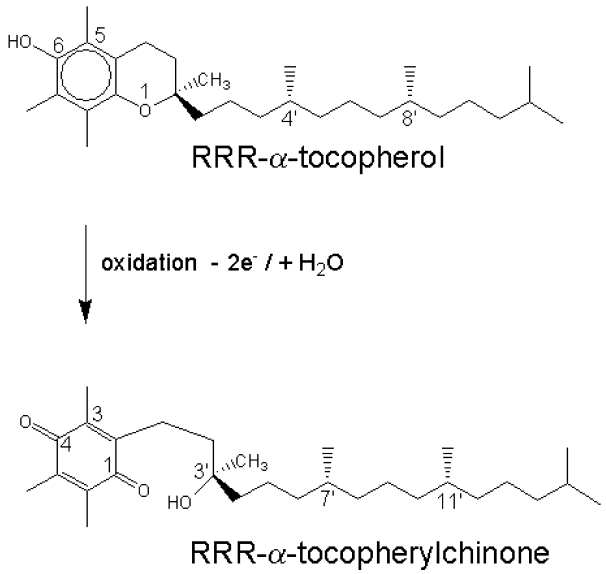

Figure 1. Flowchart describing the oxidation of RRR-α-T to RRR-α-TQ. Structure formulae are shown with the corresponding atom numbering.

SPF as tocopherol-associated protein (TAP) that binds to tocopherol has shed doubt on the role of SPF/TAP as a substrate carrier in cholesterol biosynthesis.[6]

Supernatant protein factor is an RRR-α-tocopherylquinone binder

We reported recently the crystal structure of SPF in the absence of a physiological ligand.[10] Initial attempts to prepare equimolar ligand complexes of SPF using squalene or RRR-α-tocopherol (RRR-α-T) were unsuccessful. During the course of further investigations of its substrate specificity, we unexpectedly discovered a preference of SPF for RRR-α-tocopherylquinone (RRR-α-TQ), the major physiological oxidation product of RRR-α-T (Figure 1).

Incubations of recombinant SPF with mixtures of [^{3}H]RRR-α-T and [^{3}H]RRR-α-TQ yielded a tenfold enrichment of [^{3}H]RRR-α-TQ in the protein after its separation from free ligands by cation-exchange chromatography (data not shown). In order to exclude possible artifacts, e.g. contaminations by non-specific binding of labeled compounds, SPF was incubated in parallel with equal amounts of [^{3}H]RRR-α-T and of [^{3}H]RRR-α-TQ, respectively, and its binding to the labeled compounds was analyzed by size-exclusion chromatography (Figure 2). The gel-filtration chart of the incubation mixtures reveals two major peaks of radioactivity, both corresponding to a molecular mass of 40 kDa. The radioactivity co-migrated with the protein peak, indicating a direct interaction of SPF with the labeled compounds. The ratio of the peak areas provides evidence that SPF binds [^{3}H]RRR-α-TQ with about eightfold higher affinity than [^{3}H]RRR-α-T.

Structure of the SPF–RRR-α-TQ complex

In order to answer the question of whether the observed affinity of SPF for tritiated RRR-α-TQ would be sufficient for the isolation and crystallization of its corresponding ligand complex, the

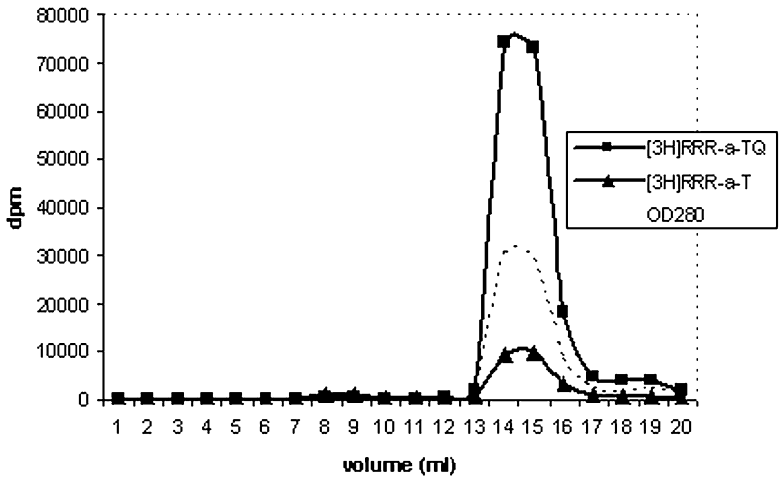

Figure 2. Elution profile of SPF ligand complexes from a Superose 12 HR gel-filtration column. For gel-filtration, 0.5 ml of recombinant SPF (1 mg/ml) was mixed in separate incubations with 15.7 μCi of [^{3}H]RRR-α-T or [^{3}H]RRR-α-TQ dissolved in 5 μl of MeOH each and stirred for 12 hours at 4 °C under nitrogen. A portion (0.1 ml) of each incubation mixture was fractionated on a Superose 12 HR gel-filtration column (Pharmacia Fine Chemicals, Uppsala, Sweden) using a 0.1 M sodium phosphate buffer (pH 7.4). Fractions of 1 ml were collected and the radioactivity of 0.5 ml of each fraction was measured by scintillation counting. A commercial calibration standard (Bio-Rad, Reinach, Switzerland) was used for the determination of elution volumes for thyroglobulin 670 kDa (9.7 ml), bovine γ-globulin 158 kDa (11.6 ml), chicken ovalbumin 44 kDa (14.1 ml), equine myoglobulin 17 kDa (15.8 ml) and vitamin B_{12} 1.35 kDa (19.9 ml). [^{3}H]RRR-α-T (25.2 Ci/mmol) was obtained from Amersham International (Buckinghamshire, UK). [^{3}H]RRR-α-TQ was prepared from [^{3}H]RRR-α-T by $FeCl_3$ oxidation following the established routes for the non-labeled compound.[11] Labeled compounds were further purified on a Nova-Pak™ C18 HPLC column from Millipore Corp. (Bedford, MA, USA) using methanol/water (96:4, v/v) as the mobile phase and stored at −20 °C under nitrogen. The radioactivity was measured with a Packard liquid scintillation analyzer (Tri-Carb 2100 TR).

Table 1. Data collection and refinement statistics

Data collection		Refinement	
Cell parameters		Resolution range (Å)	19.65–1.95
a (Å)	60.9		
b (Å)	84.6		
c (Å)	87.5		
α (deg.)	116.2		
β (deg.)	102.6		
γ (deg.)	100.2		
	Three molecules per asymmetric unit		
Space group	*P*1	No. of reflections working set	99,913
Wavelength (Å)	1.1407	No. of reflections test set	1811
No. of crystals	1	*No. of atoms*	
		Total	10,466
		Protein	9493
		Lipid	96
		No. of water molecules	877
Resolution range (Å) (outer shell)	20.0–1.93 (1.98–1.93)	*B*-Factor overall (Å^2)	21.5
No. of observations	426,429	*R* (%)	18.0
No. of unique reflections	103,050	R_{free} (%)	21.0
Completeness (%)	94.2 (71.6)	RMSD bonds (Å)	0.010
R_{sym}[a]	0.059 (0.340)	RMSD angles (deg.)	1.060
$I/\sigma(I)$	14.9 (3.2)	Ramachandran outliers[b]	18 (1.7%)

The complex of SPF with RRR-α-TQ was crystallized as described.[10] The synthesis and purification of the RRR-α-TQ diastereomer was carried out following the established routes.[11] Mixed micels were prepared by dissolving 50 mg of octyl-β-D-glucopyranoside and 2 mg of RRR-α-TQ in 1 ml of distilled water. The yellowish solution obtained was added to 5 ml of buffered SPF (2 mg/ml) containing 2 mM EDTA, 2 mM DTT, 40 mM NaCl, 20 mM Tris–HCl (pH 7.4). After incubation at room temperature for two hours, the mixture was dialysed at 4 °C, exchanging four times against 1 l of the same buffer and centrifuged for 30 minutes at 40,000***g***. The supernatant, containing the complex of SPF with RRR-α-TQ, was concentrated to 17 mg/ml by Centriprep-10 (Millipore AG, Volketswil, Switzerland). Crystals were grown at 18 °C by the sitting-drop, vapor-diffusion method. Drops were set up by mixing 1 μl of SPF-RRR-α-TQ solution with 2–6 μl of reservoir solution (containing 20% (w/v) polyethylene glycol 3350, 5% (w/v) glycerol, 200 mM sodium citrate, pH 8.3) and equilibrated against 100 μl of reservoir solution at 18 °C. Crystals were observed after one to four days and grew to an average size of 0.05 mm × 0.10 mm × 0.20 mm. Crystals were flash-cooled in a nitrogen stream at 110 K after raising the glycerol concentration of the crystallization solution in steps of 5–20% (w/v) glycerol. Data were collected at the Swiss Light Source beamline X06SA, Villigen, Switzerland and processed using XDS.[31,32] The completeness in the resolution shell 1.95–1.93 Å was low and those reflections were not used in the refinement process. The structure of the complex was refined using CNS[33] and REFMAC[34] maintaining the set of cross-validation reflections identical with that used for the native structure determination.

[a] $R_{sym} = \sum_{hkl} \sum_j I(hkl;j) - \langle I(hkl)\rangle| / (\sum_{hkl} \sum_j \langle I(hkl;j)\rangle)$, where $I(hkl;j)$ is the *j*th measurement of the intensity of the reflection with unique Miller indices *hkl* and $\langle I(hkl)\rangle$ denotes the statistical mean value of this intensity.

[b] As defined by Kleywegt & Jones.[35]

RRR-diastereomer of α-TQ was synthesized in milligram amounts from natural RRR-α-T by $FeCl_3$ oxidation as described.[11] The complex of SPF with RRR-α-TQ was prepared by incubating SPF in the presence of mixed micels consisting of octyl-β-D-glucopyranoside and RRR-α-TQ. After removing the detergent by dialysis, the RRR-α-TQ complex of SPF was crystallized using polyethylene glycol (PEG) 3350 following the established route for native SPF.[10] Attempts to prepare complexes of SPF with RRR-α-T or squalene instead of RRR-α-TQ under identical conditions followed by crystallization and X-ray analysis showed clearly that no, or only minute amounts, of ligand was bound. The crystals of the ligand complex of SPF with RRR-α-TQ diffract to a resolution limit beyond 1.95 Å and are isomorphous to those of native SPF.[10] Data collection and refinement statistics are reported in Table 1. As observed in the native structure, the monomers show some plasticity and the RMS deviations between the jelly-roll domains can be as high as 1.4 Å.

The overall structure of the SPF–RRR-α-TQ complex does not reveal significant differences compared with native SPF, whose secondary structure consists of 13 β strands, 11 α helices and eight 3_{10} helices (Figure 3). The SEC14-like domain (residues 7–244) encompasses two highly conserved structural elements, an amino-terminal three-helix coil followed by a CRAL_TRIO lipid-binding module. In contrast to the structures of SEC14[12] and α-TTP,[13] the structure of SPF contains an additional C-terminal, eight-stranded jelly-roll barrel. This jelly-roll in SPF represents the structural prototype of a sequence motif that has been discovered recently by sequence-profile analysis and been designated GOLD (Golgi dynamics) protein module with roles in Golgi function and secretion.[5] The CRAL_TRIO lipid-binding domain of the SPF–RRR-α-TQ complex consists of a central β sheet on the one side and five helices on the other side forming a hydrophobic cavity that shields RRR-α-TQ from the environment. As it is the case for the complex of α-TTP with RRR-α-T, the entrance to the cavity of the SPF–RRR-α-TQ complex is blocked by two helices (shown in pink in Figure 3) and the connecting loop. This structural element constitutes a mobile lid, as could be

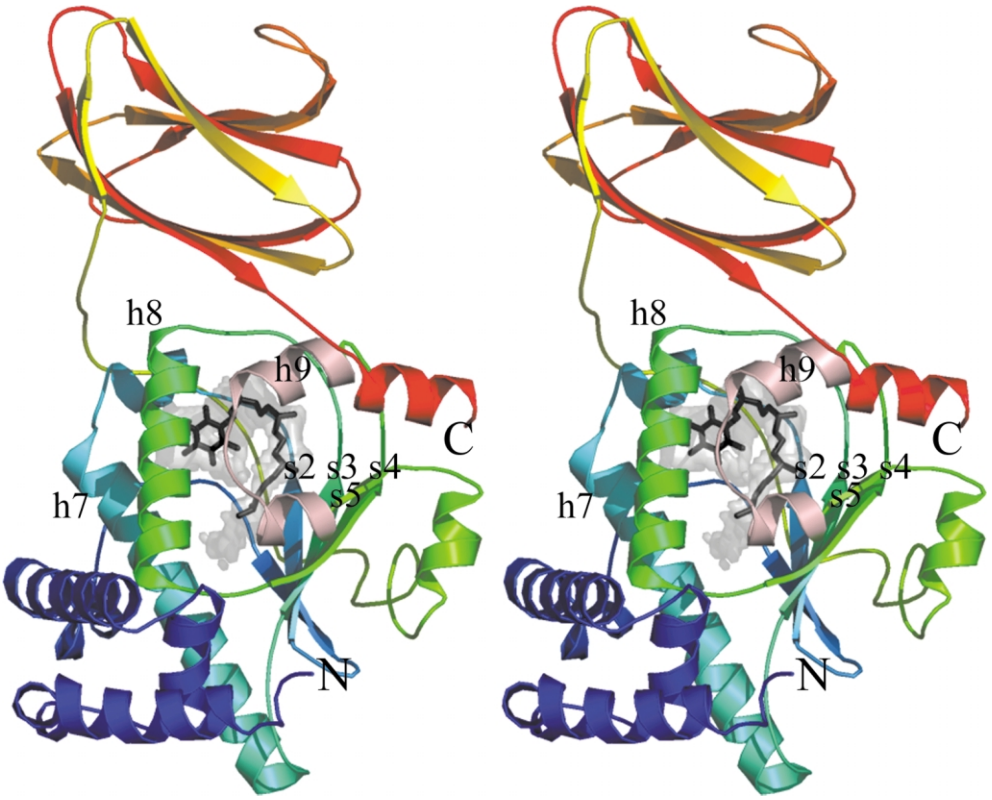

Figure 3. Stereoview of the SPF–RRR-α-TQ complex in rainbow coloring from blue (N terminus) to red (C terminus) showing the three-helix coil in dark blue, CRAL_TRIO lipid-binding domain in light blue and green, jelly-roll in yellow and red, cavity in gray (for details see Figure 5), lipid-exchange loop in pink and RRR-α-TQ in black.

shown most convincingly in the crystal structures of α-TTP with and without its ligand. Contrary to the compact cavity of α-TTP (volume 281 Å^3), however, the larger (volume 352 Å^3) cavity of SPF adopts a characteristic horseshoe-like shape found in both forms, with and without ligand, winding around two tyrosine residues (153 and 171) which are hydrogen-bonded (distance 2.7 Å) to each other (Figure 4). As already shown for α-TTP, the binding pocket does not change significantly upon ligand binding. The binding pocket of the SPF–RRR-α-TQ complex is lined mostly by hydrophobic amino acid side-chains (Figure 4). The methyl groups at positions 5 and 6 of the quinone head group are within van der Waals distance of Val167, Val108 and Leu120, while the methyl group in position 3 at the opposite side of the ring forms van der Waals contacts to Leu106, Ile103 and Tyr171. The side-chain is embedded between Tyr153 and Phe198, and ends between Phe178 and

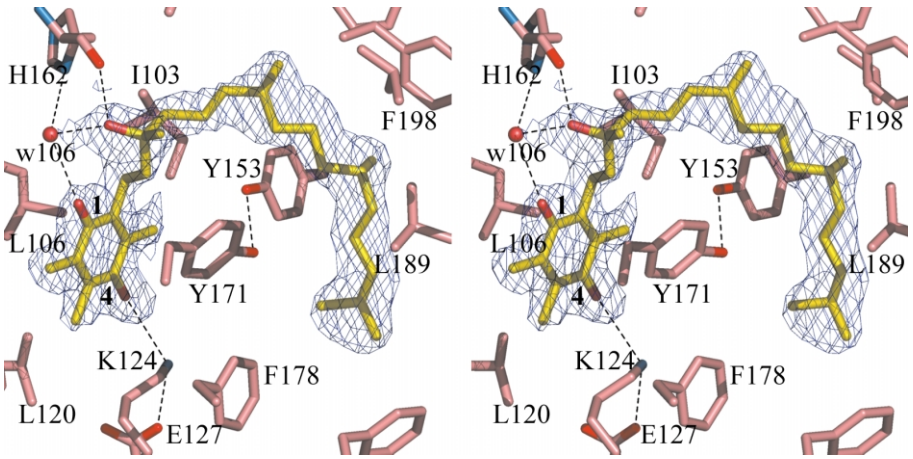

Figure 4. Electron density map of the RRR-α-TQ molecule embedded in the ligand-binding pocket of SPF. Shown is a $2F_o - F_c$ density map contoured at 1.0σ above the mean. The map was computed before RRR-α-TQ was included in the model. RRR-α-TQ is depicted as stick model in yellow (atom numbering included), internal water molecules as red balls, residues within van der Waals distance to the quinone molecule in magenta.

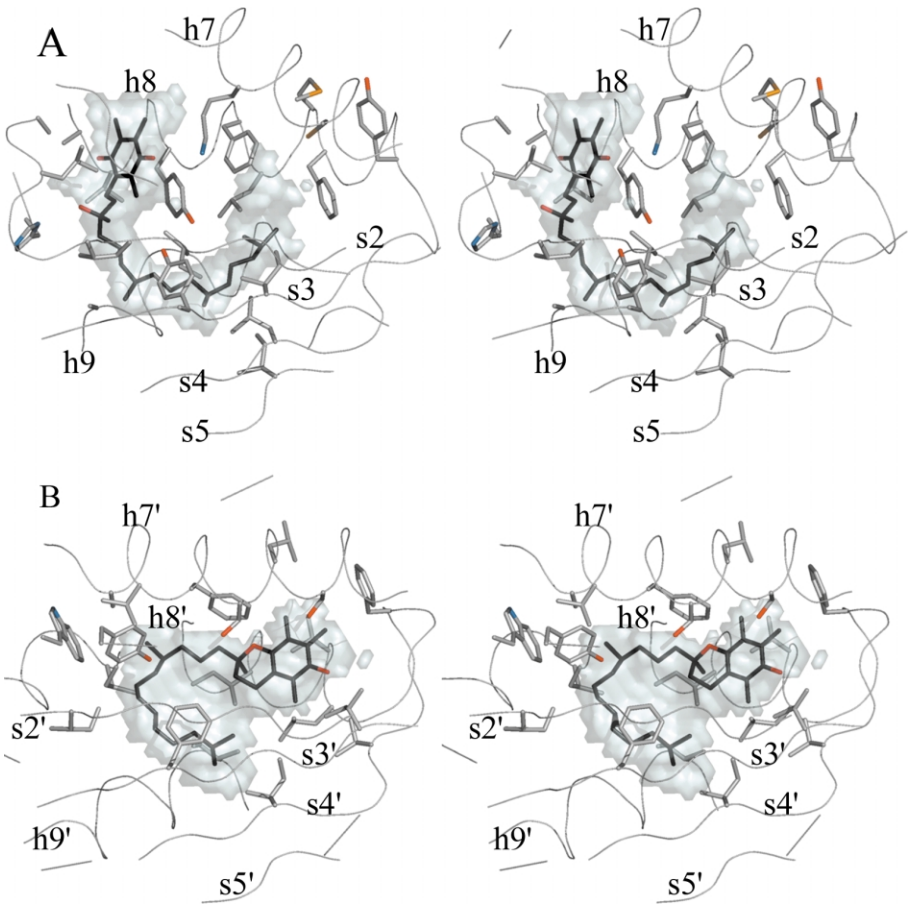

Figure 5. Stereoview of the ligand-binding pockets of: A, SPF with RRR-α-TQ; and B, α-TTP with RRR-α-T. Cavities are shown after least-squares superimposition of the C^{α} traces of the protein structures. Ligands are shown in black; side-chains in gray; oxygen, nitrogen and sulfur atoms in red, blue and orange, respectively; cavities in light gray. Cavities were calculated with the program VOIDOO.[30]

Leu189. In contrast to the rather hydrophobic cavity of α-TTP, which lacks charged residues, the RRR-α-TQ molecule of SPF is in the vicinity of Lys124, which on one side forms a salt-bridge (distance 2.9 Å) with Glu127 and is on the other side hydrogen-bonded (distance 3.4 Å) to the carbonyl oxygen atom in position 4 of the quinone head group. The second carbonyl oxygen atom in position 1 of the quinone forms a hydrogen bond *via* a water molecule to His162, whose backbone carbonyl oxygen atom is hydrogen-bonded to the C3′ hydroxyl group of the quinone molecule, which forms a second hydrogen bond (distance 2.4 Å) to the water molecule mentioned above. In analogy to the complex of α-TTP with RRR-α-T, the RRR-α-TQ molecule is sitting in the cavity of SPF (Figure 5) with the phytyl tail facing the cavity entrance and the quinone head pointing towards the interior, as can be expected for a lipid being pulled out of a micelle or bilayer. In contrast to the C^{α} traces (Figure 5) the overall positions of α-TQ in SPF and α-T in TTP are grossly different in their respective binding pockets, indicating that the ligand position in both proteins is mostly modulated by side-chain variations rather than by the backbone.

A possible physiological role for the oxidized form of vitamin E

Perhaps the most controversial aspect of the SPF structure in complex with RRR-α-TQ is its connection to the cholesterol biosynthesis pathway.[14] While it is evident that SPF enhances squalene monooxygenase activity *in vitro*,[7] direct squalene binding has not been demonstrated.[15,16] Instead, the hypothesis that SPF may influence cholesterol biosynthesis *in vivo* by RRR-α-TQ transfer between membrane compartments is intriguing and deserves consideration.

First of all it should be noted that a biological system exists that reduces RRR-α-TQ *in vivo* to RRR-α-TQH_2.[17–20] In addition, oral supplementation of humans with RRR-α-T has been shown to result in micromolar plasma levels of both RRR-α-T and RRR-α-TQH_2.[21] Plasma bound RRR-α-TQH_2 thus may serve together with other lipophilic antioxidants as defense in blocking low-density lipoprotein (LDL) (per)oxidation.[22] The concept that RRR-α-TQH_2 may act as co-antioxidant in LDL is based on the assumption that a specific carrier exists that mediates the transfer of RRR-α-TQ or RRR-α-TQH_2 to plasma lipoproteins

in analogy to α-TTP which transfers RRR-α-T to plasma very low-density lipoprotein (VLDL).[23,24] A block in LDL oxidation by RRR-α-TQH_2 would diminish the uptake of oxidized LDL by the scavenger receptor CD36, resulting in a reduction of cellular cholesterol uptake.[25] Low levels of intracellular cholesterol would then subsequently induce cholesterol synthesis *via* known cellular feedback mechanisms.[26,27]

Interestingly, recent studies by Shibata *et al*. have shown that overexpression of SPF in hepatoma cells increases cholesterol synthesis twofold and have suggested that SPF may have a role in regulating cholesterol synthesis *in vivo*.[7,28] Recent data upon the role of RRR-α-TQH_2 as natural antioxidant[29] support the idea of a putative link between the carrier function of SPF for RRR-α-TQ and the resulting regulation of cellular cholesterol synthesis. Further experiments on this group of proteins should be of considerable interest to researchers in the fields of atherosclerosis and nutrition.

Protein Data Bank accession numbers

Coordinates have been deposited with the RCSB Protein Data Bank with the accession code lolm for the structure of SPF with RRR-α-TQ.

Acknowledgements

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