THE STUDY OF LIGAND BINDING SPECIFICITIES OF THE LIPID BINDING PROTEINS: Recombinant Human α-Tocopherol Transport Protein (α-TTP), Supernatant Protein Factor (SPF) AND S. cerevisiae Sec 14p FOR VITAMIN E (RRR-α-Tocopherol) AND OTHER HYDROPHOBIC LIGANDS

A Thesis
Presented to
The Faculty of Graduate Studies of
Brock University

by
MARTA HERNANDEZ

In partial fulfillment of requirements for the degree of Master of Science September, 2003
Dedicated
To My Family
Especially My Sister Letty
ACKNOWLEDGMENTS

I greatly appreciated the opportunity to increase my knowledge and interests in research at the academic level specifically in the field of biotechnology. I am indebted to Agriculture and Agri-Food Canada for providing financial support to pursue this degree.

Special thanks to my advisor Dr. Atkinson for allowing me to join the exciting group of Vitamin E research and for keeping his door open to provide continued guidance, encouragement, support and expertise. I wish to express my gratitude to Candace for her friendship, helpful suggestions and constructive criticisms throughout this project.

Thanks to all the wonderful people at the laboratory of the Cool Climate Oenology and Viticulture Institute at Brock University.

Finally, many thanks to the most important people in my life… my mom, dad, brothers and sisters; you have instilled in me a desire to continue my pursuit for knowledge, which is the greatest gift of all. I love you very much!
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"Chemical structure of ligands tested with Sec 14p, $\alpha$-TTP, SPF/TAP and CRALBP"
ABSTRACT

One of the various functions of proteins in biological systems is the transport of small molecules, for this purpose proteins have naturally evolved special mechanisms to allow both ligand binding and its subsequent release to a target site; a process fundamental to many biological processes. Transport of Vitamin E (α-tocopherol), a lipid soluble antioxidant, to membranes helps in the protection of polyunsaturated fatty acids against peroxidative damage.

In this research, the ligand binding characteristics of several members of the CRAL-TRIO family of lipid binding proteins was examined; the recombinant human α-Tocopherol Transfer Protein (α-TTP), Supernatant Protein Factor (SPF)/Tocopherol Associated Protein (TAP), Cellular Retinaldehyde Binding Protein (CRALBP) and the phosphatidylinositol transfer protein from S. cerevisiae Sec 14p. Recombinant Sec 14p was expressed and purified from E. coli for comparison of tocopherol binding to the two other recombinant proteins postulated to traffic α-tocopherol.

Competitive binding assays using [³H]-α-tocopherol and Lipidex-1000 resin allowed determination of the dissociation constants (Kₐ) of the CRAL-TRIO proteins for α-tocopherol and ~ 20 hydrophobic ligands for evaluation of the possible biological relevance of the binding interactions observed.

The Kₐs (nM) for RRR-α-tocopherol are: α-TTP: 25.0, Sec 14p: 373, CRALBP: 528 and SPF/TAP: 615. This indicates that all proteins recognize tocopherol but not with the same affinity. Sec 14p bound its native ligand PI with a Kₐ of 381 whereas SPF/TAP bound PI (216) and γ-tocopherol (268) similarly in contrast to the preferential binding of RRR-α-tocopherol by α-TTP.

Efforts to adequately represent biologically active SPF/TAP involved investigation of tocopherol binding for several different recombinant proteins derived from different constructs and in the presence of different potential modulators (Ca⁺², Mg⁺², GTP and GDP); none of these conditions enhanced or inhibited α-tocopherol binding to SPF. This work suggests that only α-TTP serves as the physiological mediator of α-tocopherol, yet structural homology between proteins allows common recognition of similar ligand features.

In addition, several photo-affinity analogs of α-tocopherol were evaluated for their potential utility in further elucidation of α-TTP function or identification of novel tocopherol binding proteins.
1. INTRODUCTION

The absorption and physiological delivery of molecules ingested in our diet to their site of use requires the presence of distinct mechanisms that facilitate tissue specific localization. Depending on the chemical structure of the molecule, there are two significant energy barriers that molecules have to surpass -membranes and aqueous environments- for efficient absorption and delivery to the tissues and/or compartments where the molecule is required. The transport of hydrophobic compounds between cellular compartments often requires a carrier-mediated process and thus exhibits saturation kinetics and high specificity [1]. This transport is carried out by binding or transfer proteins, which enhance the selective movement of particular compounds through the body. Transfer proteins regulate the flow of material in and out of the cell and its organelles [2]. To allow for specific molecular transport, soluble transfer proteins must be able to recognize a specific molecule with high affinity, bind it, transport it and then release it at a specific destination (i.e. acceptor membrane). Some lipids and other amphipathic molecules, due to their hydrophobic nature, effectively utilize soluble proteins for transport through the cytosol. Without efficient cytoplasmic transport, these molecules would be unable to move from one membrane environment to another in a reasonable physiological timescale [1]. Several essential dietary micronutrients (i.e. vitamins A, E and K) are hydrophobic molecules that need to reach different locations in the body after ingestion. Vitamin E and the proteins involved in its metabolism are the subjects of study in this research.

1.1 Vitamin E (α-tocopherol) structure

Vitamin E is a term describing a family of structurally related compounds known as tocols. Tocols are viscous oils at room temperature and are most soluble in aprotic solvents. The tocol skeleton is composed of a polar chromanol head group and a hydrophobic phytol side chain. Only plants biosynthesize vitamin E, producing eight different tocols, four tocopherols and four tocotrienols [3]. The tocopherols possess a fully saturated phytol tail whereas the tocotrienols have double bounds at positions 3', 7' and 11' of the hydrocarbon chain characteristic of its isoprene precursor [3]. The action of a series of methyl transferases yields α-, β-, γ-, δ-tocopherols and α-, β-, γ-, δ-tocotrienols, which differ in the number of methyl groups on the chromanol ring [3] (See Fig. 1). After γ-tocopherol, α-tocopherol is the second most abundant form in nature, has the highest biological activity based on the resorption-gestation test in rats [4], and is the most efficient tocol in reversing vitamin E deficiency symptoms in humans [5]. Due to the presence of three chiral centers (2, 4', and 8') (See Fig. 1), within the phytol chain, synthetic sources of α-tocopherol produce eight stereoisomers. The mixture of compounds resulting from
chemical synthesis is designated as *all-rac-α*-tocopherol [6]. Natural α-tocopherol was shown conclusively to have the 2R, 4’R, 8’R configuration [7]. Various studies had observed that most dietary vitamin E is delivered to the liver where RRR-α-tocopherol in contrast with SRR-α-tocopherol or RRR-γ-tocopherol is preferentially delivered to tissues [8, 9].

![Chemical structure of vitamin E components]

**Fig. 1. Naturally occurring components of vitamin E**

### 1.2 Vitamin E function

The role of vitamin E as an indispensable micronutrient for the reproduction of female rats was discovered eighty years ago [10]. In humans, vitamin E deficiency primarily causes neurologic dysfunction, but the underlying molecular mechanisms are unclear [11]. The physiological necessity for vitamin E is largely described through its antioxidant activity. As a lipid soluble antioxidant, vitamin E prevents free radical damage of unsaturated lipids within cell membranes and lipoproteins [12]. Free peroxyl radicals continuously produced in living cells are efficiently trapped by hydrogen radicals donated from the phenolic hydroxyl group on the chroman ring of α-tocopherol. The hydrophobic nature of vitamin E allows stable residency in biological membranes where the vitamin is able to stabilize the membrane by forming 1:1 stoichiometric complexes with lysophospholipids and free fatty acids liberated by the action of membrane lipid hydrolysis [12].

There is a large body of evidence (and some speculation) to explain the link between the antioxidant property of vitamin E and various human pathologies. Nowadays this is being reconsidered by as yet unsubstantiated alternative functions i.e. the promotion of anti-atherosclerosis mechanisms. These beneficial non-antioxidant effects have been attributed to the
The influence of \( \alpha \)-tocopherol on the activity of enzymes like protein kinase C that are involved in lipid metabolism (i.e., chemokines: interleukin-8) [13]. However, presently there is insufficient evidence to ascribe these new roles to vitamin E without establishing a better understanding of molecular events underlying its cellular trafficking.

1.3 Vitamin E metabolism

Upon ingestion, all forms of vitamin E are absorbed in the intestine and enter the circulation via the lymphatic system. Lipoproteins facilitate their passage through the plasma and extracellular fluids by chylomicrons. During chylomicron catabolism most dietary vitamin E is delivered to the liver. In the liver only RRR-\( \alpha \)-tocopherol is incorporated and secreted into nascent very low density lipoproteins (VLDL) [11, 14] As a result of subsequent catabolism of tocopherol carrying VLDL’s, plasma and peripheral tissues become enriched in RRR-\( \alpha \)-tocopherol. Although \( \gamma \)-tocopherol is the major form of vitamin E in many plant derived oils and thus also in the North American diet, its physiological concentration is not maintained at the same level as is \( \alpha \)-tocopherol [15, 16]. Other vitamers (\( \beta \)-, \( \gamma \)- and \( \delta \)-tocopherol) are largely excreted into bile or not taken up and excreted into human waste. Parker et al recently reported that a high portion of the major dietary tocopherol, \( \gamma \)-tocopherol, is excreted in the urine as the carboxychroman metabolite 2, 7, 8-trimethyl-2-(\( \beta \) carboxyethyl)-6-hydroxychroman (\( \gamma \)-CEHC) by the P450 enzymatic mediated oxidation of the tocopherol side chain [17]. This novel water soluble metabolite has been attributed a pharmacological function as a nutriuretic compound [15]. Studies investigating the biodiscrimination between dietary RRR- and SRR-\( \alpha \)-tocopherol in rats and humans showed predominance of RRR- over SRR-isomers [18, 19]. The biological preference for RRR-\( \alpha \)-tocopherol exerted in the liver requires the existence of mechanisms within hepatocytes for physiological discrimination of the RRR-stereoisomer and \( \alpha \)-form from other ingested tocols.

1.4 Tocopherol transfer and binding proteins

The available information regarding the absorption and distribution of RRR-\( \alpha \)-tocopherol indicates the involvement of lipoproteins and specific carrier proteins for intracellular transport/distribution [14]. The lipophilicity of RRR-\( \alpha \)-tocopherol requires the assistance of an aqueous carrier to be soluble in cellular fluids. Several binding proteins have been proposed to participate in the metabolism and distribution of RRR-\( \alpha \)-tocopherol. A 32-kDa \( \alpha \)-tocopherol transport protein (\( \alpha \)TTP) present in the liver cytoplasm is suggested to regulate plasma tocopherol concentration [8, 20]. Two other proteins exhibiting more diverse tissue expression...
have been proposed to participate in the intracellular distribution of RRR-\(\alpha\)-tocopherol. A 15 kDa \(\alpha\)-tocopherol binding protein (TBP) isolated from rabbit heart cytosol has been identified but no further identification and characterization has been done on this protein since first reported 10 years ago [21]. Another recently identified 46 kDa protein was initially named “Tocopherol Associated Protein” TAP because of its ability to specifically bind tocopherol [22, 23].

1.4.1 \(\alpha\)-Tocopherol Transport Protein (\(\alpha\)-TTP) [8, 9]

Arita et al confirmed the presence of \(\alpha\)-TTP in human liver in 1995 by cloning and characterizing the \(\alpha\)-TTP gene [24]. \(\alpha\)-TTP exhibited specific binding of RRR-\(\alpha\)-tocopherol suggesting that \(\alpha\)-TTP was involved in discriminating \(\alpha\)-tocopherol from equally well-absorbed forms of vitamin E within the liver following gastrointestinal absorption of the vitamin [8, 20]. Hosomi et al examined the structural characteristics of vitamin E analogs required for recognition by \(\alpha\)-TTP [25]. An in vitro assessment of \(\alpha\)-TTP mediated inter-membrane transfer of C14-labelled \(\alpha\)-tocopherol evaluated \(\alpha\)-TTP’s ligand specificity through competition of \(\alpha\)-tocopherol transfer by \(\alpha\)-TTP using non-labelled vitamin E analogs. The relative affinities obtained correlated linearly with the known biological activities of the analogs indicated from the rat resorption-gestation test [4] (See Fig. 2). With this evidence, it was proposed that the affinity of \(\alpha\)-TTP for vitamin E analogs is the major determinant of their biological activity.

![Relative Affinities of Tocopherol Analagues](image)

Fig. 2. Correlation of the biological activities of tocopherol analogs with their relative affinities for \(\alpha\)-TTP, reproduced from Hosomi et al [25]
The crucial involvement of \( \alpha \)-TTP in the maintenance of normal plasma RRR-\( \alpha \)-tocopherol concentrations was recognized when mutations in the gene for \( \alpha \)-TTP were found in patients with familial isolated vitamin E deficiency, also called ataxia with vitamin E deficiency (AVED) [20, 26]. \( \alpha \)-TTP deficient patients showed extremely low plasma \( \alpha \)-tocopherol concentrations and suffered from neurological symptoms [14]. The presence of \( \alpha \)-TTP expression in other tissues was examined in rats. These experiments found low concentrations of \( \alpha \)-TTP in brain, spleen, lung and kidney [27, 28]. Interestingly this study suggests that the supply of RRR-\( \alpha \)-tocopherol between specialized cerebellar cortex cells is linked to \( \alpha \)-TTP expression. The underlying mechanism involved in the interaction and discrimination of \( \alpha \)-tocopherol from the diet by \( \alpha \)-TTP is not yet known.

1.4.2 Sequence similarity – Sequence homology

Protein sequence comparison is a useful tool for inferring structure and function [29]. Once a protein sequence has been determined a sequence similarity search can be done. Protein sequence similarity searches are primarily used to infer sequence homology between two or more proteins [29]. As an example a short amino acid length with high similarity is less significant than longer, lower similarities. Homologous sequences are usually similar over an entire sequence or domain and they are thought to share a common ancestor. The inference of homology is the most powerful, albeit presumptuous conclusion that can be drawn from a similarity search because homologous proteins can share similar three-dimensional structures [29]. For example, the sequence similarity and structure comparison of three members of the serine protease superfamily, bovine chymotrypsin, \( S \). \( griseum \) trypsin, and \( S \). \( griseum \) protease A, indicate that the former two proteins share strong sequence and structure similarity [29]. On the other hand the third does not share significant sequence similarity yet the protein has a very similar structure to the first two. On the contrary a high sequence similarity may not share any structural homology. Members of the lipocalin family share a very low sequence identity but display a highly conserved overall fold [30]. These examples provide an insight of the structural complexity of proteins related to their functions.

1.4.3 \( \alpha \)-TTP sequence similarity to proteins of the CRAL-TRIO family

An approach to obtain, by comparing sequence of similar proteins, additional functional clues can be offered by the use of sequence similarity searches. A BLAST search on the 278 amino acid sequence of human liver \( \alpha \)-TTP demonstrated a notable similarity to a family of lipid binding proteins known as the CRAL-TRIO family (Pfam entry: PF00650) [31]. Members of this family include the supernatant protein factor (SPF) the cellular retinaldehyde binding protein...
(CRALBP) and the yeast phosphatidylinositol transfer protein Sec 14p. Figure 3 shows a sequence alignment between these proteins.

![Alignment of proteins](alignment.png)

**Table 1.** Degree of sequence similarity between lipid binding proteins [9, 34]

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<tr>
<td>Human α-TTP to Sec 14p</td>
<td>37.4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Human α-TTP to H-SPF/TAP</td>
<td>29.0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Human SPF/TAP to Sec 14p</td>
<td>30.0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Human SPF/TAP to CRALBP</td>
<td>26.0&lt;sup&gt;b&lt;/sup&gt;</td>
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<sup>a</sup> based on the amino acid sequence from -50 to 250 of human α-TTP

<sup>b</sup> based on the amino acid sequence from 63 to 246 of human SPF/TAP
The crystal structure of human $\alpha$-TTP recently reported by Meier et al [35] illustrates that this protein folds into the prototypical Sec 14p like domain structure and confirms the common mechanism between these proteins. Two observed conformations of the structure, the closed “carrier” conformation bound to its physiological ligand (RRR-$\alpha$-Tocopherol) and the open “membrane docking” conformation bound to detergents, modelled the lipid exchange mechanism and provided the molecular basis of vitamin E retention. Either conformation is manifested by the presence of a $\alpha$-helical segment of approximately twenty three amino acids which lies at the entrance of the pocket and operates like a “lid” that affects ligand access to the binding site.

1.4.4 Supernatant Protein Factor (SPF) also referred as a TAP (Tocopherol Associated Protein)

Since the discovery of SPF/TAP more than 25 years ago it has been independently ascribed with two different functions. In 1957, a 47-kDa protein was identified in rat liver cytosol and was termed “supernatant protein factor” (SPF) due to its ability to stimulate microsomal squalene epoxidation [34]. As a first hypothesis, it was proposed that SPF facilitates the access of substrate to specific enzyme sites by interacting with the microsomal membrane [34]. This hypothesis was later excluded due to the fact that no conventional protein carrier function or squalene binding could be detected.

The same 46-kDa bovine protein was identified in 1999 by probing liver cytosol with labelled $\alpha$-tocopherol and thus it was termed the “tocopherol associated protein” (TAP). Based on SPF/TAP’s ability to specifically bind $\alpha$-tocopherol, SPF/TAP was hypothesized to be involved in cellular transport of $\alpha$-tocopherol and $\alpha$-tocopherol-dependent cell signalling [34]. A year later the human homolog of this protein was cloned and tested for its binding capacity to $\alpha$-tocopherol. By using a biotinylated $\alpha$-tocopherol derivative for an IASys resonant mirror biosensor based binding assay, the purified recombinant protein showed a binding affinity of a $K_d = 4.6 \times 10^{7}$ M for $\alpha$-tocopherol [22]. These findings supported the previously suggested function that TAP might be related to tocopherol metabolism.

Potential regulation of SPF/TAP in gene expression by $\alpha$-tocopherol was suggested through a study in cattle. Cattle were fed a diet containing increasing levels of vitamin E and the expression levels of SPF mRNA in hepatocytes were monitored over 120 days. The level of dietary vitamin E significantly increased SPF/TAP mRNA [36]. Later, Shibata et al found that TAP was identical to SPF [37] and they described its function as a “squalene transfer protein” due to the ability of the protein to enhance the activity of microsomal squalene epoxidase and promote in vitro squalene transfer between membranes [37]. A more recent study on SPF demonstrated the
relevance of phosphorylation for modulation of the protein's squalene monooxygenase activity and promotion of cholesterol synthesis [38]. Phosphorylation of SPF by ATP and protein kinases A & C6 was reported to amplify its ability to stimulate squalene monooxygenase in vitro [38].

In an attempt to resolve the functional identity of SPF/TAP, the three-dimensional structure of the human SPF was recently reported [23]. The N-terminal Sec 14p-like domain contains a horseshoe shaped ligand binding pocket and a C-terminal jellyroll [23]. This peculiar hydrophobic ligand-binding cavity is rather large (1318 Å³) and the lipid exchange loop is in a closed conformation [23]. The lipid exchange loop label refers to a helix segment defined based on the Sec 14p structure (See Fig. 4 A10) that is suggested to play an important role in membrane attachment and ligand discharge/extraction for Sec 14p. The relative position of this loop to the entrance of the binding pocket, in front or away, provides a close or open conformation. Although a bound ligand (> 16 carbon atom) was observed in the SPF/TAP crystal [23], likely originating from the host cells (E. coli) for the recombinant protein production, the compound was modelled as palmitate due to an inability to identify it by experimental means. Incubation of the protein with an excess of β-octylglucoside and phosphatidylinositol (PI) resulted in displacement of the bound ligand suggesting SPF/TAP has some affinity for phospholipids. Similar attempts to isolate ligand complexes of SPF/TAP with α-tocopherol and squalene have failed [23]. The lack of strong evidence to support a direct interaction of this protein with any of the ligands examined outlines a requirement for additional investigation of the specificities of SPF/TAP for different ligands. This research presents measurable binding affinities of SPF to these and other related ligands in attempt to characterize SPF/TAP ligand specificity and evaluate the hypothesized role of this protein in tocopherol and squalene metabolism.
1.5 *S. cerevisiae* phosphatidylinositol-transfer protein Sec 14p

Phospholipid transfer proteins (PLTPs) are ubiquitous and have been isolated from mammals and plants as well as both prokaryotic and eukaryotic microorganisms [39]. These proteins are capable of binding phospholipids and are critical to phospholipid metabolism and distribution [40]. Among well-studied PLTPs are phosphatidylinositol transfer proteins (PITPs). PITPs have a single lipid-binding site that can reversibly catalyze the transfer of either phosphatidylinositol (PI) or phosphatidylcholine (PC) between natural and artificial membranes [40]. These proteins exhibit structural and catalytic similarities including similar molecular weights and isoelectric points, and a marked preference for PI over PC [40].

The Sec 14 gene of *Saccharomyces cerevisiae* encodes a PITP protein required for the export of secretory proteins from the Golgi complex [39]. Sec 14p functions as a lipid sensor that controls the PC content of yeast Golgi membranes [39]. In *vitro* inter-membrane transfer experiments of PI and PC demonstrated a ~19-fold faster transfer rate for PI over PC by Sec 14p.
The properties of the yeast PITP are compared to those of mammalian PITPs. Although yeast PITP Sec 14p does not share primary sequence homology with mammalian PITPs, Sec 14p can restore the function of mammalian PITPs in phospholipase C signalling, exocytosis and vesicle formation [41]. Evidently, PITPs from yeast and higher eukaryotic cells are remarkably similar in their function, substrate specificity and response to alterations of membrane lipid composition [41].

The three-dimensional structure of Sec 14p was the first to be resolved from the PITPs proteins [40]. The binding site is occupied by two molecules of n-octyl-\(\beta\)-D-glucopyranoside (BOG). It was not possible to obtain a Sec 14p crystal with its physiological ligands by displacement of the two BOG molecules. In the Sec 14p crystal, the CRAL-TRIO motif forms an unusually large hydrophobic binding pocket (3000 Å\(^3\) [32]) with a lipid exchange loop in an open conformation. The lipid exchange loop is a hydrophobic helix A10/T4 (See Fig. 4) that protrudes away from the entrance of the hydrophobic pocket and is suggested to play an important role in the mechanism for Sec 14p mediated phospholipid exchange. At the opening of the pocket, a hydrogen bond network orients the two BOG molecules within the ligand-binding cavity suggestive of how Sec 14p binds PI molecules. The two BOG molecules are positioned in the structure with their head groups oriented inwards and the detergent acyl chains extended away from the Sec 14p interior [32]. The BOG head group has 4 hydroxyl groups (See Fig. 5) and all of them showed hydrogen bond interaction with the Sec 14p residues in the solved structure. Similarly, the detergent acyl chains make extensive van der Waals contacts with the extremely hydrophobic upper region of the hydrophobic pocket [32]. The structural similarity of PI (See Fig. 5) to BOG consists of a head group substituted with 5 hydroxyl groups and a hydrophobic tail. It has been proposed that BOG1 occupies the site of PI because BOG1 is bound to the lower end of Sec 14p pocket by a hydrogen network that may grant Sec 14p the specificity for PI binding (See Fig. 4). The proposed phospholipid transfer/exchange mechanism catalyzed by Sec 14p requires membrane attachment and ligand discharge/extraction by the lipid exchange loop, and ligand recognition by the head-group binding site.
1.6 Cellular Retinaldehyde-binding Protein

Active vitamin-A metabolites, known as retinoids (See Fig. 6), are essential micronutrients required for growth, differentiation, reproduction, and normal vision. The metabolic pathway of this very lipophilic compound resembles that of vitamin E. The selective movement of retinoids throughout the body is attributed to a variety of extracellular and intracellular retinoid-binding proteins [42]. Within cells, cellular retinoid-binding proteins (CRBPs) are thought to facilitate retinoid metabolism by presenting a ligand to either specific metabolic enzymes or by regulating its trafficking to tissues [42]. The biological activity of a retinoid seems to be directly related to its affinity for the CRBP [42]. Cellular retinaldehyde-binding protein (CRALBP) is a retinaldehyde-binding protein that displays high selectivity towards 11-cis-retinaldehyde [43]. CRALBP was first detected in retina about 20 years ago and shown to carry 11-cis-retinol and 11-cis-retinal as endogenous ligands [43]. CRALBP has been identified in retinal pigment epithelium and in the neural retina in the eye suggesting that this protein may be involved in the visual process [43]. Indeed, mutations in the human CRALBP gene can result in recessive retinitis pigmentosa [43]. CRALBP was also found in the pineal gland, ciliary body, cornea, optic nerve and brain [43] suggesting that this protein may have other uncharacterized functions. In vitro CRALBP serves as a substrate carrier protein for enzymes of the mammalian visual cycle for visual pigment regeneration [43]. Structure function studies have defined ligand stereoselectivity and photosensitivity [43]. Characterization of CRALBP ligand interactions with labelled retinoid gave apparent Kd values of ~21 nM for 11-cis-retinal and ~53 nM for 9-cis-retinal [43]. The
availability of this protein for our research, and its similarities with α-TTP, SPF, and PITP Sec 14p made it a good candidate for testing with α-tocopherol and 9-cis-retinal.

In summary, the characteristics that these proteins (α-TTP, SPF, PITP Sec 14p, and CRALBP) have in common are that they show amino acid sequence similarities and they also belong to the same family of lipid binding proteins defined by the homologous CRAL-TRIO domain. This domain is suggested to encompass the ligand binding pocket. These proteins are soluble proteins present in a variety of tissues with a common role, to solubilize and stabilize their hydrophobic and labile ligands in aqueous spaces and they have diverse and specific functions in regulating the disposition, metabolism and activity of their cognate ligands.

1.7 Ligand Binding

1.7.1 Protein-ligand interactions

Ligand binding sites on carrier proteins are usually depressions on the surface of the protein with the size and shape dependent upon the nature of the ligand (i.e. larger ligands are bound to larger depressions). Complementarity between the ligand and the binding site is important to support high affinity and selectivity for the ligand(s) [44]. This concept defines a steric and a physicochemical complement in which the shape of the ligand is mirrored in the shape of the binding site (steric complementarity) and molecular interactions are allowed by physicochemical complementarity between the binding site and the ligand [44]. The interactions found between a protein and its ligand are generally similar to those seen within the protein, i.e. hydrogen bonding, van der Waals interactions, hydrophobic interactions and electrostatic interactions. Hydrogen bonding is particularly important in interactions between polar groups. Proteins frequently bind ligands via complex hydrogen-bonding networks [44].

1.7.2 Binding affinity experiments:

Ligand binding experiments are important tools to demonstrate if a molecule binds to its receptor. They are commonly used in many disciplines like pharmacology, physiology,
biochemistry and molecular biology [45]. These experiments aim to describe the interactions between a receptor and its ligand by the measurements of a binding equilibrium (Equation 1)

\[
\begin{align*}
R + L & \rightleftharpoons R \cdot L \\
& \text{(Eq. 1)}
\end{align*}
\]

As opposed to kinetic experiments, equilibrium studies are used to identify thermodynamic parameters, the dissociation constant \( K_d \) and the binding capacity Bmax, and to characterize and model a receptor system. The \( K_d \) provides a numerical value that indicates the affinity of the receptor to the ligand, i.e. the smaller the value the greater the affinity. The assessment of binding affinities offers the ability to characterize the type of binding interaction between a protein and its ligand. If the affinity is low (high \( K_d \)), in physiological terms, a lack of specificity is found in comparing several ligands, thus the biological relevance of a given receptor ligand interaction may come into question. Bmax is usually defined as the stoichiometry of binding, such that Bmax = 1 for one molecule occupying the binding site [45]. Two types of equilibrium experiments are performed; saturation and competition experiments. Saturation experiments involve adding increasing concentrations of a radiolabelled ligand to a single preparation of receptor. Competitive binding involves a fixed concentration of labelled ligand that competes with increasing concentrations of a second unlabelled ligand. The unlabelled ligand can be the same compound (homologous competition) or different one (heterologous competition). Ligand binding data analysis must be done with a defined model relating the binding interaction to the data for interpretation by graphical methods. Scatchard plots are common for the analysis of ligand binding data however several analogous methods have been developed ultimately derived from the work of Cheng & Prusoff (Equation 1) [46]. The Prism Graphpad 4.0 program with homologous and heterologous competitive binding with ligand depletion (one site model) was used for the data analysis in this research.

The study of ligand binding affinities of lipid binding proteins, recombinant human \( \alpha \)-Tocopherol Transport Protein (\( \alpha \)-TTP), Supernatant Protein Factor (SPF), Cellular Retinaldehyde Binding Protein (CRALBP) and S. cerevisiae Sec 14p for vitamin E (RRR-\( \alpha \)-tocopherol) and other hydrophobic ligands are presented in this research. This thesis provided critical data for interpreting the ligand binding specificity of \( \alpha \)-TTP and similar proteins having the CRAL-TRIO binding motif.
2. MATERIALS AND METHODS

2.1 Materials

Tetrabutylammonium fluoride (TBAF) was purchased from Aldrich (Oakville, ON). Imidazole, isopropyl β-D-Thiogalactopyranoside (IPTG), ampicillin, kanamycin, agar, bio-tryptone, tryptone, and yeast extract all purchased from BioShop (Burlington, ON). Ethyl acetate, butanol, tetrahydrofuran, ether, hexane, methanol, etc., solvents of high purity or reagent grade were obtained from Caledon Laboratories (Georgetown, ON). Cetyltrimethyl ammonium bromide (CTAB) was purchased from ICN Biomedical (Aurora, OH). His-Trap Nickel column purification kit was obtained from Amersham Pharmacia (Sweden). Glycine and scintillation fluid were supplied by Fischer Scientific. Bradford protein assay, bovine serum albumin (BSA), and SDS-PAGE low range standard were obtained from BioRad. MBI Fermentas provided GeneRuler 1kb and 100bp DNA ladder. QIA quick Gel extraction kit was purchased from Qiagen. Lipidex (hydroxyalkoxy propyl dextran) substituted with 10% by weight with alkyl chains of length C15–C18 was purchased from Canberra Packard or Sigma. Guanidine di- and triphosphate, Triton X-100, lysozyme, δ-tocopherol, trolox, cholesterol, oleic acid, trans-retinol, retinoic acid, α-tocopheryl acetate, α-tocotrienol, α-tocopheryl quinone, squalene, β-octo-glucoside, were supplied by Sigma (St. Louis, MO). α-Tocopherol was prepared in lab from the commercial acetate, β-tocopherol was a gift from Cognis, Inc. USA, and γ-tocopherol was isolated from soy oil distillates (Esai, Co.). Tissue derived bovine liver phosphatidylinositol and phosphatidylcholine were obtained from Avanti Polar Lipids. The tetrafluorazidoaryl photoaffinity ligands (C6-C9) were synthesized by H.Lei Organic Chemistry Lab Brock University [47].

\[^{3}H\]α-tocopherol (51.6 Ci/mmol) was prepared by Amersham-Pharmacie Life Sciences as the tert-butyl-dimethylsilyl-α-tocopherol (TBDMS-α-toc). \[^{3}H\]squalene (30 Ci/mmol) was obtained from American Radiolabeled Chemicals Inc., and \[^{3}H\]α-tocopheryl quinone (51.6 Ci/mmol) was prepared in our laboratory from the oxidative reaction of \[^{3}H\]α-tocopherol [48]. All other reagents were of molecular biology grade.

2.1.1 Bacterial Strain, Plasmids, Proteins and Restriction Enzymes

The S. cerevisiae Sec 14p gene (CAA89225.1) cloned into pQE-31 with 5 and 3-SalI restriction sites was provided by SunGene GmbH & Co, (Gatersleben, Germany). This construct placed the Sec 14p gene under the control of the transcription rate promoter T5. The T5 promoter is repressed by the Lac repressor during cell growth and induced after with IPTG, which inhibits the Lac repressor. The DNA provided also contained pREP4 plasmid due to the expression strain
used for DNA isolation. The pREP4 plasmid encodes for the Lac repressor protein. SG13009 and JM105 *E. coli* cells were purchased from Novagen. SG13009 also contained the pREP4 plasmid. Dr. Danny Manor, Division of Nutritional Science, Cornell University provided several fusion TAP/SPF proteins derived from different plasmids. The proteins were engineered as a C-term His-tagged unclipped, and N-term-tagged, tagged removed (See Table 2). Dr. Sanders, University of Texas at Austin provided two more SPF/TAP constructs; SPF/TAP-38 protein with a deletion of 76 base pairs nucleotide and SPF/TAP-46 of a normal size His-tag protein. C. Panagabko provided the recombinant human α-Tocopherol Transport Protein (α-TTP). Protease Inhibitor tablets, RNase A, BamH1, KpnI, PvuII, EcoR1, HindIII, Sall, PstI, NdeI and Sure/Cut buffer H generic enzyme buffer were obtained from Boehringer Mannheim.

<table>
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<th>Tag location</th>
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### 2.2 DNA Extraction and Protein Expression

The pQE-31/Sec 14 and residual pREP4 DNA was used to transform the competent SG13009 and JM105 *E. coli* cells according to the following protocols:

- All cells were propagated in Luria Britani media
- Media for JM105 cultures was supplemented with 100 µg/mL ampicillin (LB amp) to select for pQE31/Sec 14p transformants and SG 13009 cells were grown in the presence of 100 µg/mL ampicillin and 30 µg/mL kanamycin (LB amp-kan) for selection of cells containing both pREP4 and pQE31/Sec 14p.
- Large scale competent cells preparation, transformation and expression were preceded by selection of single colonies for small-scale pre-cultures and LB plating.

#### 2.2.1 *E. coli* competent cells protocol

A pre-warmed 100 mL of LB medium was inoculated with 1 mL of overnight pre-culture of SG13009 *E. coli* cells and incubated in a shaker at 37°C – 230 rpm until an OD 

00 = 0.5 was reached. Then the culture was placed on ice for 5 min and cells were collected by centrifugation at 4000 g (4°C – 5 min). The supernatant was discarded and cells were kept on ice. The cells were gently re-suspended in 30 mL of 4°C TFB1 buffer (45 mM MnCl₂·4H₂O, 10 mM CaCl₂·2H₂O, 100 mM KCl, 3 mM hexaminocobalt chloride, 10 mM MES pH 6.3) and incubated on ice for 90
min. Another round of centrifugation followed and the pellet was re-suspended in 4 mL of ice-cold TFB2 buffer on ice. Competent cells were used fresh or frozen in aliquots of 100 – 200 µL in ethanol bath and stored at −80°C.

2.2.2 Transformation Protocol

30 µL of the competent SGI3009 E. coli cells allowed to thaw on ice were added to 2 µL plasmid DNA (pQE-31/Sec 14p + pREP4) in a 1.5 mL sterile eppendorf tube. This mixture was left on ice for 40 min and heat shocked in a 37°C water bath for 50 sec. Subsequently, the cells were cooled on ice for 2 min. To allow time for the shocked cells to build resistance, 300 µL of LB was added and the suspension was incubated for 20 min at 37°C. Aliquots of this culture were plated on LB antibiotic amended plates and allowed to incubate overnight at 37°C.

2.3 DNA extraction and purification

Freshly transformed SGI3009 E. coli cells were screened to confirm the insertion of the cloned plasmid into the cell line by restriction analysis. The integrity of the insert was further verified by DNA sequencing (Norgen Biotek).

2.3.1 Mini-preparations protocol

1.5 mL of an overnight pre-culture was placed in an eppendorf tube and centrifuged at 13000 g for 5 min. The supernatant was drawn off and the cells were re-suspended in 200 µL STET buffer (8% sucrose, 0.1% triton X-100, 50 mM EDTA, 50 mM Tris pH 8.0) by vortexing. 5 µL of 50 mg/mL lysozyme solution was added and the sample incubated for 15 min after vortexing for few seconds. The eppendorf tube was then placed in a boiling water bath for 45 sec and centrifuged for 15 min at room temperature. The resulting pellet was removed with a pipette tip and discarded. 8 µL of 5% cetyltrimethylammonium bromide (CTAB) was added to the supernatant, the solution mixed and then centrifuged for 5 min at 13000 g. The supernatant was discarded and the pellet was re-suspended in 300 µL of 1.2 M NaCl. 750 µL of ice cold 95% ethanol was added followed by centrifugation for 5 min at 13000 g. The supernatant was decanted and the pellet was washed with 800 µL of 70% ethanol and centrifuged for 2 min. The washed pellet was dried under vacuum in a SpeedVac for 10 min at low setting. Finally the pellet was re-suspended in 20 µL Milli-Q (MQ) water and stored at −30°C. The left over pre-culture at the beginning of the mini-preps was used to prepare freeze cultures (1:1 mixture of overnight pre-culture and 50% glycerol) to be store at −80°C. An agarose gel was run to confirm the presence of the extracted DNA.
2.3.2 DNA agarose gel protocol

A 1 % gel was prepared with 0.5 g of agarose and 50 mL of 1 X TBE buffer (0.045 M Tris-borate, 1 mM EDTA) by micro waving the suspension for 1 min on high power to dissolve the agarose. Once the flask was cool enough to be held by hand (T ~ 50°C) 2 µL of 10 mg/mL ethidium bromide was added and mixed. The gel was poured into the assembled gel box and let it solidified for more than 15 min covered. The gel was placed in an electrophoresis chamber filled with 1 X TBE buffer after the comb was removed. According to sample concentration to run approximately 1 – 3 µg; 1-10 µL sample was prepared for electrophoresis with 3 µL 10 X loading buffer (10 mM EDTA, 0.015 % bromophenol blue, 0.015 % xylene cyanol FF, 10 % glycerol, 10 mM Tris – HCl [pH 7.6]), and water to a total volume of 14 µL. Samples and 2 µL of 1 kb or 100 bp DNA ladder marker were loaded. The gel was run until marker was near the edge (~ 1 hour) at 100 V. A photograph of the gel under UV was taken using the BioRad gel doc system.

2.3.3 DNA purification protocol

1 – 2 µL of 4 mg/mL RNase A was added to the re-suspended DNA from the mini-preps for each 20 µL sample and left to sit at room temperature for more than 30 min. One tenth of the sample volume of sodium acetate 3.2 M pH 5.0 was added followed by 2 X the sample volume of ice-cold 95 % ethanol. This mixture was allowed to precipitate at −30°C for more than 20 min and centrifuged at 13000 rpm for 5 min. The supernatant was decanted and the pellet dried. The purified DNA was re-suspended in MQ before restriction enzyme digestions or storage at −30°C.

2.3.4 DNA restriction enzyme digestion protocol

Digestions were set up in 1.5 mL clean eppie tubes. For a total volume of 21 µL, calculations were done to determine the amount of MQ to be added according to the sample volume + 2 µL of Sure cut buffer and last 1 µL of enzyme. Samples were placed at 37°C to digest for more than 7 hours. The digested DNA was then run on an agarose gel and a DNA gel extraction procedure was done to prepare the Sec 14p gene for sequencing.

2.3.5 QIA quick gel extraction protocol

The DNA fragment was excised from the gel using a clean sharp scalpel. Removing extra agarose minimized the gel size. The gel slice was weighed and 3 X volume of QG buffer (solubilization and binding buffer that contains chaotrophic salts and a pH indicator) was added to one volume of gel and incubated at 50°C for 10 min. After the gel was completely dissolved and the color of the mixture did not change (yellow for optimum pH 7.5 for membrane efficiency) the solution was applied to the column and centrifuged (1 min – 1300 rpm). The flow through was discarded and another 0.5 mL of QG buffer was added to the column to remove any traces of agarose and centrifuged for one minute. To wash the column 0.75 mL of PE buffer (contains
chaotropic salts) was added, incubated for 5 min and centrifuged for a minute. Another round of centrifugation followed and then the DNA was eluted from the column with 30 \( \mu \)L of MQ or EB buffer (10 mM Tris.Cl, pH 8.5) into a clean 1.5 mL microfuge tube. The elution buffer (EB) was applied to the center of the column left to stand for 1 min and centrifuge for another minute. The purified un-digested and digested (921 bp fragment) DNA samples were sent to Norgen Biotek Corporation for sequencing.

2.3.6 DNA quantification protocol

Two types of methods widely used to measure the amount of nucleic acid in a preparation were used. DNA was determined spectrophotometrically measuring the sample absorbance at 260 nm and 280 nm in a quartz cuvette. The reading at 260 nm allowed the calculation of the concentration (\( \mu \)g/mL) of nucleic acid in the sample using the formula [DNA] = Absorbance at 260 nm/0.02; where 0.02 is the absorbance of 1 \( \mu \)g/mL of DNA solution at this wavelength. The ratio between the readings at 260 nm and 280 nm provided an estimate of the purity of the DNA. Pure preparations of DNA give ratio values of 1.8 to 2.0.

The ethidium bromide fluorescent quantification of the amount of DNA was done by comparing the UV-induced fluorescence yield of the sample with that of a series of known concentration standards.

2.4 Expression of recombinant \textit{S. cerevisiae} Sec 14p in \textit{E. coli} SG 13009

200 mL of LB amp kan medium was inoculated with 2 mL of an overnight pre-culture (100 X dilution) and allowed to grow at 37\( ^\circ \)C in a 220 rpm shaker until an OD_{600} = 0.6 was reached. Protein expression was induced by addition of IPTG to achieve a final culture concentration of 1mM IPTG and allowed to proceed for 5 – 15 hrs at 37\( ^\circ \)C – 220-rpm shaker. Small aliquots of un-induced and induced controls were sampled and stored at –80\( ^\circ \)C.

2.4.1 Cell lysis and harvesting of protein

The induced culture was centrifuged at 13000 rpm (4\( ^\circ \)C – 15 min). The supernatant was discarded and the cell pellet was re-suspended in 5 – 20 mL of start buffer (10 mM imidazole, 500 mM NaCl, 20 mM phosphate buffer pH 7.4) supplemented with protease inhibitors to a final concentration of 0.1mM. A solution of 50 mg/mL of lysozyme was added to a final lysozyme concentration of 100 \( \mu \)g/mL. This solution was allowed to sit at room temperature for 30 min before sonication. Multiple rounds of 30 – 40 sec pulsed sonication bursts were repeated on ice until cells appeared lysed as indicated by reduced sample viscosity, darkening of the lysate and lower sonication pitch. The lysate was centrifuged at 13000 rpm (4\( ^\circ \)C – 30 min) to remove cell
debris. The supernatant was kept as a native soluble protein in start buffer at 4°C until purification.

2.4.2 Basic purification protocol for Hexahistidine-tagged proteins

Column preparation: a new 1 mL HiTrap chelating column was washed with 5 mL of Milli-Q water (MQ), filled with 0.5 mL of 0.1 M NiSO4 and washed again with another 5 mL of MQ. A 5 mL disposable syringe and leur adaptors were used for loading the solutions to the column.

Column equilibration: 10 mL of start buffer was passed through the column.

Sample application: the supernatant was loaded on the column with an approximate flow rate of 1 – 4 mL/min. The flow through fraction was collected and the column was washed with another 10 mL of start buffer. The wash fraction was also collected. The protein was eluted with 5 mL of elution buffer (400 mM imidazole, 500 mM NaCl, and 20 mM phosphate buffer pH 7.4) and collected in 1 mL fractions. Fractions were stored at 4°C until later use. After protein elution, a wash with 10 mL of start buffer regenerated the column. The protein concentration of elution fractions was determined using the calculated extinction coefficient of the recombinant Sec 14p fusion protein (His-Sec 14p) and the Bradford assay.

For protein determinations using the extinction coefficient the absorbance at 280 nm of 1 mL of an elution fraction or the diluted sample in start buffer was measured. The absorbance of a blank (buffer alone) was also determined and subtracted to the sample absorbance. Sample concentration was calculated using the calculated extinction coefficient for recombinant Sec 14p (40,590 cm⁻¹ molar⁻¹) [49].

The Bradford assay was performed with a diluted (10 μL sample in 90 μL distill water) or concentrated protein sample in 2 mL of Bradford working reagent. The sample was left at room temperature for 5 min before the absorbance was read at 595 nm. Protein concentration was extrapolated from a calibration curve of BSA in elution buffer over the range of 25 – 400 μg/mL. The purity and concentration of the eluted protein from the metal affinity column were confirmed by SDS-PAGE.

2.4.3 Poly-acrylamide SDS-PAGE Gel electrophoresis protocol

12.5 – 15 % resolving (2.3 mL of distill water, 1.5 mL of 1.5 M Tris pH 8.8, 2.3 mL of 40% Acryl/bis, 60 μL of 10% SDS, 12 μL of 50% glycerol, 2.5 μL of TEMED, 75 μL of 10% APS) and 5.5 % stacking (1.3 mL of distill water, 0.5 mL of 0.5 M Tris pH 6.8, 0.3 mL of 40% Acryl/bis, 10 μL of 10% SDS, 4 μL of 50% glycerol, 2.5 μL of TEMED, 30 μL of 10% APS) gels were poured and let to polymerize according to the recipe [50]. The poured or pre-cast gel was assembled on the gel apparatus as indicated by the manufacturer. 30 μL samples were mixed
with 10 μL (1:4 dilution) of 6 X sample buffer (0.5 M Tris pH 6.8, 0.4 % SDS, 30 % glycerol, 0.1 % DTT, 0.02 % bromophenol blue) in an eppendorf tube and heated for 2 min at 90°C. A marker was also prepared with 2 μL of SDS-PAGE low range standard; 10 μL distill water and 10 μL of 6 X sample buffer. Samples and marker were loaded into the gel and run for approximate 3 hrs at 70 V. The gel was stained with Coomassie brilliant blue solution (100 mg of Coomassie brilliant blue G-250 in 50 mL of 95% ethanol, 100 mL of 85% phosphoric acid in a liter of water) for more than an hour. The stained gel was de-stained in 7.5% HOAC – 5% MeOH for few hours photographed using BioRad gel doc system.

2.5 Deprotection of Photoaffinity labels

Four photoaffinity analogs of α-tocopherol were available. The compounds possessed a tetrafluorobenzyl moiety separated from the chromanol ring by varying hydrocarbon chains from C₆ to C₉ (See Fig. 7). During the synthesis of the tetrafluoroazidoaryl photoaffinity labels the phenol of the chromanol ring was protected as the tert-butylidimethylsilyl (TBDMS-) ether.

The protected TBDMS – C₆, C₈, C₉ tetrafluoroazidoaryl photoaffinity labels were re-suspended in dichloromethane and an aliquot was transferred to tared vials to roto-evaporated and dried on high vacuum. Between 1.8 – 2.7 mg of the protected photoaffinity label were deprotected in 200 μL tetrahydrofuran (THF) using 5 μL TBAF (Tetramethylammonium fluoride). Before TBAF was added the sample was sitting on ice for 10 – 15 min. After shaking, the reaction was allowed to proceed for approximate 2 hrs. To monitor the completeness of the reaction TLC plates in hexane - ethyl acetate (5:1) were run before and after TBAF addition. Sample visualization on the TLC plate was achieved by staining the plate with a solution of 4 % sulfuric acid in methanol. At the end of the reaction 15 μL of 1N HCl in methanol was added to neutralize any TBAF excess. Samples were dried with nitrogen and re-suspended in 200 μL hexane. The samples were purified in Pasteur pipet silica columns in 10:1 hexane – ethyl acetate.
Small silica columns were prepared for each reaction. The purified label was eluted with 5:1 hexane – ethyl acetate in approximate 0.5 mL fractions. Between 16 to 18 fractions were collected and every second fraction was checked by TLC to determine where the pure compound eluted. The spotted fractions were transferred into a clean, pre-weighed vial and evaporated by roto-vap and high vacuum until dryness. Photolabels were reconstituted in an appropriate volume of absolute ethanol to obtain a desired concentration of ~ 3 mM for the binding assays. TLC plates of the final products were also run to confirm the purity and presence of the de-protected photoaffinity label. A small aliquot of each compound was submitted for GC-MS analysis. The C₇ tetrafluoroazidoaryl label was available de-protected requiring several silica column purifications as above prior to use.

2.6 Deprotection of [³H]-α-tocopherol

Storage of the [³H]-α-tocopherol as the tert-butyldimethylsilyl- (TBDMS-) ether required de-protection prior to use. TBDMS-α-tocopherol was deprotected in dry tetrahydrofuran (THF) on ice using a large molar excess of tetrabutylammonium fluoride (TBAF) and subsequently purified on a silica column after acidification with 1 N HCl. Purified fractions were evaporated and re-suspended in absolute ethanol to allow addition of one or more µL to the binding assays. Purity of the [³H]-α-tocopherol was assessed by TLC before use (> 80%). Using unlabelled α-tocopherol for identification, the TLC plate was sectioned after development in 10:1 hexane:EtOAc and the radioactivity in each segment counted in the scintillation counter.

2.7 Preparation of [³H]-α-tocopheryl Quinone [48]

The [³H]-α-tocopheryl quinone was prepared by oxidation of approximately 800-nmol α-tocopherol in 50 % ether, 45 % methanol, and 5 % water with 2 µmol FeCl₃ for 1.5 hrs at room temperature. The quinone was purified from the reaction mixture by silica chromatography (5:1 hexane:EtOAc) and selected fractions were evaporated and re-dissolved in ethanol. Purity of the quinone was 90 % as verified by TLC prior to use.

2.8 Binding Assays

Homologous and heterologous competition curves with [³H]-α-tocopherol (51.6 Ci/mmol); [³H]-squalene (30 Ci/mmol) and [³H]-α-tocopheryl quinone (51.6 Ci/mmol) were used to determine ligand dissociation constants by adaptation of the Lipidex assay previously reported by Timmers et al [51]. Briefly this assay relies on the application of an equilibrated aqueous sample containing [³H]-α-tocopherol and protein to a Lipidex column. The hydrophobic matrix
retains all free tocopherol and allows the protein bound tocopherol to be collected. After washing with incubation buffer (25 mM Tris-HCl, 0.1M KCl, 100 μM Triton-X100, 1mM EDTA), the free tocopherol is eluted with 100% methanol into a fresh vial yielding two fractions; bound (aqueous) and free [\(^3\text{H}\)]-α-tocopherol (methanol) for each sample. Each fraction is equalized for solvent quenching differences and the cpm counted in a Beckman LS1600 scintillation counter.

Assays were conducted with 1.8 – 2.5 nM [\(^3\text{H}\)]-α-tocopherol and 0.4 – 0.8 μg Sec 14p or 5 – 10 μg TAP protein in a reaction volume of 320 μL. Samples were incubated at 37°C for 2 hrs and placed on ice for 10 minutes after which the separations on Lipidex column were performed at 4°C to “freeze” the equilibrium. Only 250 μL was applied to the Lipidex column. Unlabelled competitor was added to achieve 8 – 10 points spanning the concentration range of roughly 1.8 nM to 20 μM of ligand. All competitors except phospholipids were added from 100% ethanol stocks to maintain a concentration of ethanol in the final assay below 1%.

All assays were performed in triplicate in at least two separate experiments. Competition with α-tocopherol was run with each new ligand as a positive control. Assays with α-tocopherol were also performed with 0.2 μg of the bacterial cell lysate without the recombinant protein as a negative control. In addition, samples without any added protein were used to assess non-specific binding.

2.8.1 Phospholipid Ligands

Phosphatidylinositol and phosphatidylcholine supplied in chloroform were dried by removing solvent under nitrogen and leaving the samples overnight under high vacuum. The lipid cakes were re-suspended in incubation buffer by sonicating the suspension on ice several ~10 sec pulses until a transparent solution was achieved. This stock solution of phospholipid vesicles was used to performed binding assays in a modified fashion. Protein-free control incubations were run for each point of the competition curve. Thus, the hot tocopherol that flowed through the Lipidex column under the protein-free controls was subtracted from that observed in the presence of protein.

2.9 Data Analysis

Normalized bound cpm of [\(^3\text{H}\)]-α-tocopherol was entered into Prism Graphpad 4.0. Counts per minute data were transformed to account for solvent quenching and the reaction volume counted. Transformed data was regressed using either homologous (for α-tocopherol) or heterologous competition (assuming one binding site) for all other ligands. All equations are based on the formulations of Cheng and Prusoff for a one binding site model [46]. The resultant dose response curves of cpm vs. log [ligand] (M) all yielded correlation coefficients greater than
0.92 and Bmax values within 85 – 100 % of the assayed protein concentration. All results are presented as the average ± standard error of the mean (SEM).
3. RESULTS

3.1 Cell line transformation and gene sequencing

SunGene (GmbH & Co. KgaA, Gatersbelen, Germany) provided DNA containing the S. cerevisiae Sec 14 gene cloned into pQE-31 (pQE31/Sec 14p) along with the pREP4 plasmid due to the expression strain used for DNA isolation. The pQE31/Sec 14p placed the Sec 14p gene under the control of the T5 promoter based transcription-translation system. The pQE31/Sec 14p construct was engineered to produce a fusion protein of Sec 14p with an N-terminal hexahistidine affinity tag. The pREP4 plasmid encodes for the Lac repressor protein to suppress transcription of the pQE31/Sec 14p gene by the host polymerase at the T5 promoter in the absence of IPTG. IPTG is considered a strong inducer and its function is to inhibit the Lac repressor that acts at the T5 promoter [50]. When IPTG is added to a grown culture the Lac repressor protein is deactivated and the T5 promoter de-repressed and thereby activating gene transcription.

The DNA provided containing both plasmids was used to transform the E. coli cell lines JM105 and SG13009. The JM105 host strain possesses Lac9 mutation that confers this strain the capacity to produce higher levels of the Lac repressor; which would decrease the leakiness of protein expression under non-induced conditions. Very likely the amount of Lac repressor produced by JM105 would not be enough for the strong T5 promoter. When we received the DNA there was no information describing the selective marker for the pREP4 plasmid. As such, it was not possible to select for JM105 transformants containing both the pQE31/Sec 14p and pREP4 plasmids. The identification and recovery of pQE31/Sec 14p transformants was independently selected by their resistance to ampicillin conferred by the β-Lactamase gene (bla) present in pQE31.

The transformation of the JM105 cell line with the cloned DNA produced a low efficiency outcome of 4 – 5 colonies per petri plate on selective medium. The presence of the pQE31/Sec 14p (4385 bp) construct in the transformed colonies was assessed through isolation of the recombinant DNA and gel electrophoresis (See Fig 8). The isolated DNA was also digested with the PstI, NdeI and SalI restriction enzymes to re-confirm the recovery of both plasmids. Three fragments were expected after PstI digestion of the DNA based on the presence of two cleavage sites within the pREP4 plasmid (2817 bp and 923 bp) and no digestion of the pQE31/Sec14p plasmid. DNA digestion with NdeI was also expected to produce three fragments. NdeI has no sites within pREP4 (3740 bp) but cuts once within pQE31 and once in the Sec 14p gene to produce 2818 bp and 1567 bp fragments from pQE31/Sec 14p. If pREP4 were present SalI would generate two bands from the pREP4 plasmid (1490 – 2728 bp) plus another two from the clone (921 and 3464 bp). The digested samples were run on an agarose gel and the expected
pREP4 bands were not observed indicating the absence of the pREP4 plasmid in the transformed JM105 cells (See Fig. 9).

After these preliminary findings another host strain was chosen to achieve higher transformation efficiencies and tighter regulation of protein expression. The SG13009 E. coli competent cells were transformed with the recombinant DNA (pQE-31/Sec 14p + pREP4). SG13009 carry the pREP4 plasmid and use of this cell line is suggested when proteins are poorly expressed in other systems [52]. The observed transformation efficiency of this cell line was ten times higher than the JM105. Following transformation, DNA was extracted and a gel was run to confirm the isolation of both plasmids (See Fig. 10). It is evident from Figure 10, that the yield of both plasmids pQE31/Sec 14p (4385 bp) and pREP4 (3740) are low based on the band intensities observed, when loading the same DNA concentration (0.1 – 0.2 μg) as the JM105 transformed cell line. These plasmids are low copy number and we estimated an overabundance of pREP4 from the cell line and the recombinant DNA provided. As illustrated in Figure 11, digestions of the isolated DNA with Sall produced the expected fragments for pREP4 (2738 – 1490 bp) and the
clone (3464 – 921 bp). Since the Sec 14p gene was cloned into pQE31 using SalI, digestion with SalI produced fragments corresponding to the pQE31 plasmid and the gene encoding the Sec 14p (921 bp). The purity of the DNA was determined by the spectrophotometric method [50] prior to sequencing. Due to the presence of two plasmids in the isolated DNA, two types of DNA samples were prepared and sent for sequencing; the isolated miniprep DNA containing both plasmids and the digested gene fragment (921 bp). The 921-bp fragment was extracted and purified from an agarose gel. A higher yield of the 921 bp fragment was obtained from the transformed JM105 E. coli cells compared with the SG13009 transformants (See Fig. 11). Sequencing of the S. cerevisiae Sec 14p gene confirmed the integrity of the insert.

![Fig. 10. DNA from E. coli SG13009 host strain containing pQE31/Sec 14p gene + pREP4](image1)

![Fig. 11. Sall digested SG13009 DNA sample and Sec14p gene fragment (921 bp) from JM105, arrows show digested bands](image2)

### 3.2 Protein Expression and purification

Successful transformation of the Sec 14 pQE-31/pREP4 gene into SG13009 E. coli cell line produced a 37.4 kDa fusion protein featuring an N-terminal 6His-tag. This constitutes 2.5-kDa addition to the 34,900 Da native protein. Preliminary expression trials performed by SunGene found large amounts of protein within four hours induction at only one temperature, 37°C. The expressed protein was mostly found in the insoluble fraction and approximately 1/10th was found in solution. The protein recovery in the soluble fraction was improved by us to ~ 2/10th with the increase of induction time to 7 hrs or more. The transformed cells were cultured on a
0.15 to 0.25L scale, which produced a cell pellet with an average weight of 1.7 g. Metal affinity purification of the soluble cell fraction under native conditions yielded approximately 0.5 – 1 mg of relatively pure protein (50 – 80% purity) as calculated by comparison of the protein concentration and visual inspection of the fractions by SDS-PAGE. Trace impurities bands were visible on a SDS-PAGE gel shown in Figure 12. A detailed calculation of the yield from one expression system is presented in Table 3.

![SDS-PAGE gel](image)

**Fig. 12.** SDS-PAGE gel basic protocol for protein purification

<table>
<thead>
<tr>
<th>Table 3. Total protein yields throughout purification of the recombinant Sec 14p from 150 mL culture of <em>E. coli</em> SG13009 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td><strong>Total Protein (mg)</strong></td>
</tr>
<tr>
<td>Un-induced bacterial lysate</td>
</tr>
<tr>
<td>Induced bacterial lysate</td>
</tr>
<tr>
<td>Insoluble fraction</td>
</tr>
<tr>
<td>Soluble fraction</td>
</tr>
<tr>
<td>Proteins un-retained on metal affinity</td>
</tr>
<tr>
<td>Proteins washed from column with start buffer</td>
</tr>
<tr>
<td>Eluted protein from soluble fraction</td>
</tr>
<tr>
<td>Yield of His-Sec 14p</td>
</tr>
</tbody>
</table>

(a) % of total protein from the induced bacterial lysate

Fusion proteins can be purified directly from bacterial lysates and are recovered from the matrix under mild elution conditions that preserve antigenicity and functionality of the protein [53]. Histidine-tagged proteins can be desorbed from HiTrap chelating with buffers containing imidazole. A balance between the amount of imidazole needed to prevent non-specific binding of contaminants and the amount of imidazole needed to elute the histidine-tagged protein is
important. When large amounts of protein are needed and high yield is more important than optimum purity a basic purification protocol is used. The basic purification protocol requires the use of only one high concentration of imidazole (i.e. 400 mM) to elute the recombinant protein. On the other hand the purity optimization protocol entails the use of a stepwise elution of the protein from the gel using buffers containing increasing concentrations of imidazole [53]. First trials of the binding assays were run with larger amounts of protein 3 – 4 μg for competitive and non competitive assays. These initial tests needed higher yields of protein, which tolerated the use of reasonably pure protein preparations (50 – 80% purity) initially obtained with the basic purification protocol. Figure 12 shows an SDS gel of purified Sec 14p with the basic purification protocol; band impurities are observed on the eluted protein fraction # 2. Competition curves using these protein concentrations (3 – 4 μg) yielded dissociation constants, Kd’s, higher (~ 1 μM) than expected. Reported values in the literature for lipid binding proteins that belong to the CRAL-TRIO family are in the nM range (i.e. CRALBP binding affinity to its native ligand 11-cis-retinal has a Kd = 21 nM [43]). The high dissociation constants observed and a high percentage of [3H]-α-tocopherol bound to the protein in the assays suggested that conditions for the binding assays such as protein and ligand concentration range may be too high. Consequently, assays were conducted using smaller amounts of Sec 14 protein, less than 1 μg, and the ligand concentration range was expanded to include low nM concentrations to be able to estimate the lowest possible Kd values. Since less protein was required for these later assays, efforts were made to increase the purity of the recombinant protein with the use of the purity optimization protocol. The stepwise elution of Sec 14p from the Lipidex column with buffers containing increased imidazole concentrations is illustrated in Figure 13. On Figure 13 it can be observed a significant improvement on the number of band impurities in the eluted protein fraction # 2.

The same binding degree (~70%) was observed on assays performed with protein from both purification schemes, the optimized purification and the initial non-gradient elution, suggesting that the impurities observed on the basic purification protocol did not have any influence on the binding behavior. The only consideration taken when using protein from the higher yielding sample purification scheme was in the calculation and determination of the amount of protein needed for the assays. The amount needed was higher for a low purity protein. An alternative purification protocol was designed, as a compromised between the two previously described protocols, in which the 10 mL start buffer wash was broken down into three small washes of 10, 40 and 100 mM imidazole concentration. This alternative protocol produced a protein fraction with the purity comparable to the optimized purification protocol.
Further studies were done to determine if the observed binding was a product of the impurities eluted with the protein fraction in the basic purification protocol. Thus, binding assays of the un-induced, flow through (proteins unretained on the Ni column during sample application), and wash solutions from the protein purification were assayed for $[^3]$H-$\alpha$-tocopherol binding and compared to the eluted protein fraction. There was not any significant binding of these solutions in contrast to the purified protein (See Table 4). This experiment also demonstrated that any of the trace impurities observed in the Sec 14p eluted fraction by SDS-PAGE do not contribute to the binding phenomena. One point binding assays were also performed with other two proteins, lysozyme and bovine serum albumin, low and higher molecular weight proteins respectively, which did not show any appreciable binding to $\alpha$-tocopherol when assayed at the same or greater molarity as Sec 14p (See Table 5). This negative control confirmed the reliability of the binding protocol.

**Table 4. Comparison Study of the binding of Sec 14p and protein expression and purification solutions to $\alpha$-tocopherol**

<table>
<thead>
<tr>
<th>[Ligand] µM</th>
<th>Sec 14p</th>
<th>flow through</th>
<th>wash</th>
<th>un-induced</th>
<th>control</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>60.3</td>
<td>16.1</td>
<td>19.3</td>
<td>2.7</td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>49.1</td>
<td>8.6</td>
<td>14.2</td>
<td>3.1</td>
<td>10.7</td>
</tr>
<tr>
<td>1.0</td>
<td>4.5</td>
<td>1.6</td>
<td>1.9</td>
<td>5.2</td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>% Bound</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>------------------</td>
<td>---------</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sec 14p</td>
<td>81.02</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-TTP</td>
<td>45.83</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lysozyme</td>
<td>2.20</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BSA</td>
<td>2.57</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No protein</td>
<td>2.56</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

3.3 Protocol 1: binding assays with high amount of protein

As a preliminary phase, kinetic studies were performed to characterize each variable of our system. Optimum conditions for assay parameters such as incubation time and temperature were determined for subsequent use in the binding experiments. Equilibrium was achieved in one hour and at 37°C. These parameters were determined by taking aliquots of an incubation mixture (label ligand + protein + non-label ligand) at different time intervals until equilibrium was reached. This was done at room temperature and at 37°C. The binding experiment consisted in the incubation of calculated amounts of cold and hot ligands with the receptor/protein for the already determined time and temperature to reach equilibrium. After achieving equilibrium, the mixture was chilled on ice for ten minutes and an aliquot passed through a Lipidex column at 4°C. From the column separate fractions of bound and free were eluted with buffer and methanol respectively. Each fraction was then equalized, added scintillation cocktail and the radioactivity (cpm) counted on a scintillation counter.

Prior to conducting competition experiments, preliminary tests were performed consisting of competitive and non-competitive binding assays with different concentrations of protein from 0.25 – 4.0 μg, a constant amount of [³H]-α-tocopherol (∼ 4 nM) and a set concentration of cold ligand (α-tocopherol) accordingly to make it competitive or non-competitive. For the non-competitive assay the set concentration of cold ligand was 0.2 μM and a set up of a 100-fold excess (20 μM) of cold α-tocopherol made the competitive assays. Results demonstrated the unexpected binding capacity of *S. cerevisiae* Sec 14 protein for α-tocopherol in both types of assays (See Fig. 14 and 15). Graphical results of the competitive binding assays show the effect of non-specific binding phenomena with the use of large amounts of cold ligand. In the absence of protein it can be observed at the 0 μg of protein concentration column on Figure 15. These graphs also illustrate that a maximum percentage of ligand bound (> 50%) by Sec 14p is reached between 2 to 4 μg of protein.
Having noted that Sec 14p does actually bind α-tocopherol and based on the results of the preliminary studies competitive binding curves were conducted parallel to α-TTP with 3 μg of protein and 2 - 4 nM [3H]-α-tocopherol. Unlabelled competitor was added to achieve 9 - 11 points spanning the concentration range of 0.002 to 40 μM. Additionally, calculations of the proportional amount of protein to the label and non-label ligands based on its specific activity and concentration added confirmed that the ratio protein:ligand was 7:1 having more protein than ligand which was a desirable requirement for the assays. Binding curves were obtained and $K_{d}$s
were calculated with the Prism Graphpad 4.0 program. The calculated $K_d$s were in the $\mu$M range, a 1000-fold higher than expected nM values for $\alpha$-TTP affinity to $\alpha$-tocopherol (See Fig. 16). $K_d$s for lipid binding proteins of the CRAL-TRIO family reported in the literature are in the nM range (i.e. CRALBP binding to its native ligand 11-cis-retinal has a $K_d$ of 21 nM [43]).

![Graph showing binding curves to various tocols](image)

**Fig. 16.** 3 $\mu$g $\alpha$-TTP binding curves to $\alpha$, $\beta$, $\gamma$ & $\delta$-tocopherols, $\alpha$-tocopheryl acetate & squalene. The curves show that calculated $K_d$s are in the $\mu$M range.

It was also observed from these results that Sec 14p was binding to all the hydrophobic ligands (tocols and non tocols) tested and with similar affinity, thus, there was no indication of any selectivity or discrimination. Table 6 illustrates similar bound percentages of Sec 14p to $\alpha$- and $\gamma$-tocopherol. Taken together, all these results suggested that the parameters set for the experiment were not correct and did not meet requirements such as only a small fraction (equal or less than 50%) of ligand (label and un-label) binds the receptor for using the Prism Graphpad 4.0 Program.
Table 6. 3 µg Sec14p + [³H]-α-tocopherol competitive binding assays and other unlabeled ligands

<table>
<thead>
<tr>
<th>[Cold Ligand] (µM)</th>
<th>α-tocopherol</th>
<th>γ-tocopherol</th>
<th>Oleic acid</th>
<th>Squalene</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>90.78</td>
<td>91.34</td>
<td>91.87</td>
<td>91.98</td>
</tr>
<tr>
<td>0.0025</td>
<td>90.94</td>
<td>90.51</td>
<td>81.66</td>
<td>87.7</td>
</tr>
<tr>
<td>0.025</td>
<td>84.72</td>
<td>89.82</td>
<td>74.04</td>
<td>79.6</td>
</tr>
<tr>
<td>2.5</td>
<td>58.28</td>
<td>73.17</td>
<td>72.49</td>
<td>69.46</td>
</tr>
<tr>
<td>5</td>
<td>41.56</td>
<td>48.93</td>
<td>67.17</td>
<td>62.24</td>
</tr>
<tr>
<td>10</td>
<td>26.12</td>
<td>20.90</td>
<td>63.56</td>
<td>53.72</td>
</tr>
<tr>
<td>20</td>
<td>6.42</td>
<td>10.49</td>
<td>46.25</td>
<td>41.48</td>
</tr>
<tr>
<td>39.75</td>
<td>4.80</td>
<td>4.13</td>
<td>29.27</td>
<td>17.42</td>
</tr>
</tbody>
</table>

3.4 Protocol 2: binding assays with low amount of protein

A new experimental design was planned to use lower amounts of protein and the smallest feasible concentration of label ligand to estimate the lowest possible $K_d$ values. The underlying theory for this new approach resided on the avoidance of large amounts of bound ligand to the protein, which affects the free ligand concentration and the non-specific binding in each assay thus complicating the simple relationship between $EC_{50}$ and $K_d$ in the equilibrium. Sec 14p was assayed at 0.2, 0.4, 0.6, and 0.8 µg and a constant concentration of [³H]-α-tocopherol, 1.8 nM. Results are shown on Table 7. From this it was established that an appropriate amount of Sec 14 protein to be use for the binding assays was 0.4 – 0.8 µg.

Table 7. Determination of S. cerevisiae Sec14p ligand binding per µg of protein (reported as % bound)

<table>
<thead>
<tr>
<th>[Ligand] µM</th>
<th>0.0</th>
<th>0.2</th>
<th>0.4</th>
<th>0.6</th>
<th>0.8</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.3</td>
<td>39.2</td>
<td>61.6</td>
<td>67.3</td>
<td>74.8</td>
<td></td>
</tr>
<tr>
<td>8.4</td>
<td>43.8</td>
<td>41.5</td>
<td>66.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>29.6</td>
<td>19.1</td>
<td>23.8</td>
<td>22.3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Generally the easiest method for solubilizing hydrophobic molecules such as α-tocopherol is adding non-ionic or zwitteronic detergents. Triton X100, a non-ionic detergent, was used to maintain the solubility of the lipophilic ligand and minimize loss of the ligand to surfaces. The detergent was added to the incubation buffer at a concentration of 100 µM and an extra volume (additional 100 µM) was added to the working ligand solution when preparing stocks that had greater than 40 µM tocopherol because of solubility problems observed in which the solution turned cloudy. First trials of the new design were done using additional detergent for preparation of the working ligand solution and competitive binding binding assays were run. The calculated $K_d$ of Sec
14p from these assays for α-tocopherol was ~ 100 nM. For the new experimental design the addition of extra detergent was not needed due to the lower concentrations (up to ~ 40 μM) of cold α-tocopherol used. The 100 μM detergent added to the incubation buffer was sufficient to support these concentrations of α-tocopherol, which was observable by visual inspection of the solution that did not turn cloudy. Since the extra amount of detergent was no longer required, another set of assays was done without additional Triton X100. The $K_d$ for α-tocopherol binding to Sec 14p obtained from these experiments was three times higher (~ 300 nM), indicating lower affinity for α-tocopherol. The extra detergent was displacing the binding curve to the left resulting in a higher apparent affinity of the protein for the ligand. These findings finally lead to the proper protocol for the binding assays using a range of cold ligand of 4.5 nM - 20 μM, lower protein (< 1 μg) and no additional Triton X100 over the 100 μM in the incubation buffer.

The lower amount of protein to be used also raised another concern, an accurate way to determine low protein concentrations. Bradford assays were the analytical method chosen since the beginning and this method was acceptable for large amounts of protein. For low determinations (< 1 μg), this assay becomes less reliable since small errors in the determination have a larger impact when small amounts of protein are required [54]. The calculated extinction coefficient for protein concentration determination was used as an alternative to confirm the Bradford assay results. An extinction coefficient of 40,590 cm$^{-1}$ molar$^{-1}$ was calculated for Sec 14p fusion protein at a pH of 6.5 [49]. Protein concentration was commonly determined using both methods. In general, the extinction coefficient calculation yielded a 10% higher protein concentration than the Bradford assays. Unfortunately neither method provided complete assurance of the protein concentration.

**3.5 S. cerevisiae Sec 14p binding to various hydrophobic ligands**

Table 8 summarizes the dissociation constants obtained for recombinant *S. cerevisiae* Sec 14p to 16 hydrophobic ligands in comparison to the binding data for the recombinant human α-TTP and CRALBP determined by C. Panagabko.
Table 8: Comparison of dissociation constants of recombinant human α-TTP, *S. cerevisiae* Sec14p, SPF, and CRALBP for various hydrophobic ligands with *in vitro* transfer abilities of tocopherols by rat α-TTP and with the relative activities of tocopherols in the rat fetal resorption assay.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Dissociation Constant (nM)*</th>
<th>Relative Affinity of TTP for α-tocopherol b</th>
<th>Rat-Resorption Gestation Assays c</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>α-TTP</td>
<td>Sec14p</td>
<td>SPF/TAP</td>
</tr>
<tr>
<td>α-tocopherol</td>
<td>25.0 ± 2.8</td>
<td>373 ± 89</td>
<td>615 ± 15</td>
</tr>
<tr>
<td>β-tocopherol</td>
<td>124 ± 4.7</td>
<td>3914 ± 286</td>
<td>393 ± 32</td>
</tr>
<tr>
<td>γ-tocopherol</td>
<td>266 ± 9</td>
<td>3990 ± 420</td>
<td>268 ± 13</td>
</tr>
<tr>
<td>δ-tocopherol</td>
<td>586 ± 75</td>
<td>3908 ± 900</td>
<td>731 ± 82</td>
</tr>
<tr>
<td>SRR-α-tocopherol</td>
<td>545 ± 62</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Trolox</td>
<td>1004 ± 126</td>
<td>ic</td>
<td>nd</td>
</tr>
<tr>
<td>α-tocopheryl acetate</td>
<td>1639 ± 89</td>
<td>5559 ± 900</td>
<td>nd</td>
</tr>
<tr>
<td>α-tocopheryl succinate</td>
<td>526 ± 54</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>6-O-carboxymethyl-α-tocopherol</td>
<td>879 ± 65</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>α-tocotrienol</td>
<td>214 ± 13</td>
<td>4726 ± 884</td>
<td>nd</td>
</tr>
<tr>
<td>α-tocopheryl quinone</td>
<td>814 ± 86</td>
<td>5701 ± 395</td>
<td>441 ± 4</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>ic</td>
<td>ic</td>
<td>nd</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>7200± 1030</td>
<td>ic</td>
<td>ic</td>
</tr>
<tr>
<td>Squalene</td>
<td>ic</td>
<td>ic</td>
<td>879 ± 123</td>
</tr>
<tr>
<td>n-β-octyl glucopyranoside</td>
<td>ic</td>
<td>ic</td>
<td>nd</td>
</tr>
<tr>
<td>phosphatidylinositol</td>
<td>1415 ± 106</td>
<td>381 ± 43</td>
<td>216±31</td>
</tr>
<tr>
<td>phosphatidylcholine</td>
<td>nd</td>
<td>6123 ± 234</td>
<td>1183±178</td>
</tr>
<tr>
<td>9-cis retinal</td>
<td>786 ± 67</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>trans-retinol</td>
<td>ic</td>
<td>ic</td>
<td>nd</td>
</tr>
<tr>
<td>retinoic acid</td>
<td>ic</td>
<td>ic</td>
<td>nd</td>
</tr>
</tbody>
</table>

(a) all data is expressed as the average ± SEM  
(b) (25)  
(c) (4)  
nd = not determined; ic = incomplete competition, competition was not achieved at 20 μM [ligand]

The ligands tested were: the tocopherols α-tocopherol, the native ligand of α-TTP, the three other forms β-, γ-, δ-tocopherol and one tocotrienol (α-). Three derivative compounds of tocopherol (α-tocopheryl acetate, α-tocopheryl quinone and Trolox) were also tested. Other ligands included cholesterol, oleic acid, squalene, retinoic acid and trans-retinol.

Phosphatidylinositol (PI), *S. cerevisiae* Sec 14p’s native ligand, and phosphatidylcholine (PC) were also tested.
Fig. 17. Sec 14p - α-tocopherol competitive binding curve

Fig. 18. Sec 14p – other ligands competitive binding curves
As illustrated in Figures 17 & 18 and Table 8, Sec 14p binds almost all the tocopherols with varying affinities, except Trolox, while showing a strong preference for α-tocopherol (Kₐ of 373 nM). The Kₐs for β-, γ- and δ-tocols were ten times higher (3.9 μM) indicating low affinity. α-Tocotrienol and α-tocopheryl acetate and quinone showed Kₐs from 4.7 to 5.7 μM. Incomplete competition is observed for Trolox, cholesterol, oleic acid, squalene, n-β-octyl glucopyranoside, trans-retinol and retinoic acid. The ratio of the dissociation constants obtained for Sec 14p with PI – PC, (381:6123) displays a ~16 – fold higher preference for PI over PC. This is consistent with a 19 – fold higher preference observed with Sec 14p in an earlier report [39]. Figure 19 shows the binding curves of Sec 14 to these ligands.

Fig. 19. Sec 14p – PI and PC competitive binding curves
The Sec 14p affinity obtained for its native ligand PI ($K_d = 381$ nM) was comparable to that obtained for $\alpha$-tocopherol. This unusual result was attributed to possible complications in the assays by the presence of phospholipid vesicles. Phospholipid solutions were prepared as small unilamellar vesicles in incubation buffer by sonication for use in the binding assays. The phospholipid vesicles could interfere in the measurement of the affinities of phospholipids to $\alpha$-TTP and Sec 14p with the Lipidex assay by encapsulating the [3H]-$\alpha$-tocopherol within the vesicles and altering the bound and free counts. A steady high percentage bound was observed along all the points on the competitive binding curve. Previously reported studies to demonstrate Vitamin E integration into membranes through preparation of model membrane systems consisting of phospholipid dispersed in aqueous media and co-dispersion of $\alpha$-tocopherol had shown that $\alpha$-tocopherol inserts into the phospholipid molecules that form a spontaneous assembly of pseudo-bilayers [12]. With this consideration in mind, PI and PC binding assays were performed with protein-free controls for each point in the competition curve to account for the formation of tocopherol phospholipid complexes alone. Thus, the hot tocopherol that flowed through the Lipidex under the protein-free controls was subtracted from that observed in the presence of protein.

### 3.6 Human Supernatant Protein Factor (SPF)/Tocopherol Associated Protein (TAP) binding assays

Supernatant protein factor (SPF)/tocopherol associated protein (TAP), Sec 14p and $\alpha$-TTP belong to the same family of lipid binding proteins sharing a common domain; the CRAL-TRIO motif. The recombinant human SPF/TAP provided by Dr. Manor (Division of Nutritional Sciences, Cornell University) was also tested in the first experimental design using 3µg in each assay. This fusion protein had a C-terminal hexahistidine tag. Because of our expectation that SPF might bind to tocopherol much better than it did, the initial amount of SPF/TAP tested was also switched to lower quantities (< than 1 µg) as indicated in Protocol 2 (See section 3.4 page 34). In both cases (high and low amount of protein), neither of these quantities used showed any appreciable binding to $\alpha$-tocopherol (See Fig. 20) even though SPF/TAP was identified through its ability to bind [3H]-$\alpha$-tocopherol.
Higher amounts of protein > 3 μg were not tested based on the pre-established idea that non-specific binding increases with increased amounts of protein, making it more difficult to identify specific binding phenomena. Thus, the possibilities that the sample of SPF/TAP received contained a very small fraction of functional protein and/or SPF was a low affinity protein that required higher amounts were not fully explored at this point. Characteristically, low affinity proteins use larger amounts of protein to establish the equilibrium and to produce reliable data.

Previously reported SPF binding to tocopherol was experimentally described as done with prolonged incubation times. This raised the possibility that the observed binding could be the result of SPF/TAP binding to oxidized forms of [3H]-α-tocopherol. Metabolism studies reported in the literature have found that α-tocopheryl quinone metabolite is a main hepatic oxidation product of α-tocopherol antioxidant function [11]. In our experimental work we have observed that during prolonged exposure at room temperature of solutions of [3H]-α-tocopherol readily oxidized to the quinone. This chemical instability was monitored by TLC purity checks and confirmed by TLC runs beside freshly synthesized α-tocopheryl quinone standard. Thus, an experiment was designed to explore the possibility of SPF binding to oxidized products of tocopherol due to long incubation times. A low quantity of SPF/TAP (0.4 μg) was incubated with 2 nM [3H]-α-tocopherol for either 2 hours as in the regular assay or overnight, prior to performing Lipidex separations. Identical experiments were also performed with Sec 14p and α-TTP for comparison. Surprisingly SPF/TAP appeared to bind more tocopherol (See Fig. 21) as
compared with the other two proteins Sec 14p and α-TTP suggesting that SPF/TAP may have appreciable affinity for oxidation products of α-tocopherol. It was also observed that α-TTP binding diminished and this correlated well with the extent of oxidation of the tocopherol evident by TLC.

![Graph](image)

**Fig. 21. Two hour and overnight incubation of [H]-α-tocopherol with α-TTP, SPF/TAP and Sec 14p**

The next step was to prepare [H]-α-tocopheryl quinone through iron catalyzed oxidation of [H]-α-tocopherol [48]. TLC assessments in 5:1 Hexane:EtOAc indicated that the quinone was produced in quantitative yield (100% conversion) as compared to a α-tocopheryl quinone standard. Approximately 2 - 4 nM [H]-α-tocopheryl quinone with 90% purity, determined by TLC and radioactivity count, was used to run competitive assays with SPF. A dissociation constant of 441 nM was obtained for the binding affinity of SPF/TAP to the quinone (See Table 8).
Previous reports suggested the presence of a GTP binding motif within the SPF/TAP sequence [34]. Considering this possibility, binding assays were performed with the addition of 2 mM nucleotides (GTP, GDP), and 5 mM cations (Mg\(^{2+}\), Ca\(^{2+}\)) in various combinations as possible modulators for SPF/TAP. None of these supplementations modulated SPF/TAP’s affinity for α-tocopherol (See Table 9).

<table>
<thead>
<tr>
<th>Modulator</th>
<th>[mM]</th>
<th>% Bound of (^{3})H-α-tocopherol</th>
</tr>
</thead>
<tbody>
<tr>
<td>No modulator</td>
<td>0.0</td>
<td>10.05</td>
</tr>
<tr>
<td>Calcium (Ca)</td>
<td>5.0</td>
<td>10.55</td>
</tr>
<tr>
<td>Magnesium (Mg)</td>
<td>5.0</td>
<td>13.11</td>
</tr>
<tr>
<td>Guanidine diphosphate (GDP)</td>
<td>2.0</td>
<td>13.28</td>
</tr>
<tr>
<td>Guanidine triphosphate (GTP)</td>
<td>2.0</td>
<td>20.34</td>
</tr>
<tr>
<td>GDP – Ca</td>
<td>2.0 - 5.0</td>
<td>9.51</td>
</tr>
<tr>
<td>GDP - Mg</td>
<td>2.0 - 5.0</td>
<td>24.91</td>
</tr>
<tr>
<td>GTP - Ca</td>
<td>2.0 - 5.0</td>
<td>20.98</td>
</tr>
<tr>
<td>GTP - Mg</td>
<td>2.0 - 5.0</td>
<td>13.42</td>
</tr>
</tbody>
</table>

At this point implementation of larger amounts of protein in the binding assays was suggested to determine if the sample of recombinant SPF/TAP was a mixture of functional protein with a large proportion of misfolded functionally inactive protein. It was decided that binding assays would be performed using 0.6, 0.8, 1.0, 5.0, 10, and 20 µg to establish if higher specific binding could be demonstrated with higher protein concentrations. To address the possibility that the location of fusion sequences perturbed the order or magnitude of ligand affinities Dr. Mannor also provided a number of recombinant SPF/TAP proteins (See Table 2). The recombinant proteins received were CPDM 1-1 with 4 amino acid N-terminal addition after thrombin cleavage (394 total amino acids), CPDM 1-3 with a T7 tag (14 amino acids) N-terminal that remained after thrombin clipping (404 total amino acids), and IDM3 with C-terminal non-clippable his-tag (22 amino acids addition to a total of 412 amino acids and 48.5 kDa size). These constructs were tested at the six different protein concentrations. In addition two other constructs, SPF/TAP – 38 and SPF/TAP – 46 received from Dr. Sanders (Department of Molecular Genetics and Microbiology, University of Texas at Austin) were also analyzed. SPF/TAP – 38 was a 76 base nucleotide deletion protein at the 5’ coding DNA strand giving rise to a 25 amino acids C-terminal deleted protein with final 365 amino acids and 38 kDa size. SPF/TAP – 46 was a normal size C-terminal his-tag (22 amino acids) protein for a total of 412 amino acids and 48.5 kDa size. \(^{3}\)H-Squalene was also used to determine the binding affinity of all these proteins for
this ligand since SPF has also been reported to stimulate squalene monooxygenase potentially through binding of squalene [38]. All proteins were first assayed at a single ligand concentration with both tritiated ligands and significant binding (~50 - 75%) of [³H]-α-tocopherol was observed at 5, 10 and 20 μg for the C-terminal constructs and SPF/TAP - 38 and 46 (See Table 10). That was not the case for the amino terminal constructs, CPDM 1-1 and 1-3, that showed 17 – 30% bound at 5 and 10 μg and a maximum of 40 – 55% bound at 20 μg of protein.

| Protein Sample | α-T | 0.6 | 0.8 | 1.0 | 5.0 | 10.0 | 20.0 | α-T | SQ | α-T | SQ | α-T | SQ | α-T | SQ | α-T | SQ |
|----------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| IDM3           | 11.1| 15.3| 20.0| 20.0| 18.0| 21.0| 29.2| 50.4| 44.5| 65.2| 48.3| 75.0| 54.6|
| CPDM 1-1       | 5.3 | 6.3 | 19.0| 4.6 | 19.3| 5.7 | 13.6| 21.3| 22.9| 30.3| 21.3| 40.1| 37.8|
| CPDM 1-3       | 8.4 | 15.5| 23.9| 11.2| 21.5| 8.0 | 20.0| 17.0| 29.9| 30.0| 39.8| 54.9| 48.9|
| SPF/TAP - 38   | 37.8| nd  | 44.1| nd  | 50.6| nd  | 80.4| nd  | 84.8| nd  | 91.0| nd  |
| SPF/TAP - 46   | 17.6| nd  | 21.7| nd  | 35.0| nd  | 59.7| nd  | 76.9| nd  | 83.6| nd  |

nd = not determined

Three points of a competitive assay were done with the C-terminal constructs, SPF/TAP-38 and 46 for the three protein concentrations 5, 10 and 20 μg (See Table 11). It was evident that the binding was the result of the protein affinity for the ligand and not an artifact of non-specific binding. Then duplicates of seven point competition assays were conducted to obtain the dissociation constants of these proteins for α-tocopherol and squalene (See Table 8). Both, SPF/TAP – 38 & 46 produced a higher K_d (~ 1.5 μM) for α-tocopherol compared to the one obtained with the IDM3 construct (615 nM). Further testing was done with the C-terminal construct, IDM3. 20 μg of the N-terminal constructs, CPDM 1-1 and 1-3, were also tested in a 3 points competition (See Table 11) and produced an insufficient percentage bound (< 50%) to obtain a reliable binding curve, thus no further testing was done with these recombinant proteins. The criteria used to consider a binding assay to be successful was the observation of sufficient binding, 50% or more, at the lowest possible amount of protein. In order to establish a complete binding isotherm the competitive binding curve will have to descend from 90 to 10% specific binding over an 81-fold increase in the concentration of the cold competitor ligand.
Table 11. Three point concentration competitive binding assays of recombinant SPF/TAP proteins with α-tocopherol (reported as % bound)

<table>
<thead>
<tr>
<th>µg of protein</th>
<th>Recombinant SPF/TAP proteins</th>
<th>µg of protein</th>
<th>Recombinant SPF/TAP proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>10</td>
<td>20</td>
<td>5</td>
</tr>
<tr>
<td>0.0</td>
<td>46.5</td>
<td>56.5</td>
<td>69.6</td>
</tr>
<tr>
<td>1.5e-7</td>
<td>46.4</td>
<td>51.1</td>
<td>65.7</td>
</tr>
<tr>
<td>1.0e-5</td>
<td>18.7</td>
<td>19.9</td>
<td>38.7</td>
</tr>
</tbody>
</table>

nd = not determined

The final dissociation constants of SPF/TAP binding to squalene and α-tocopherol are also reported in Table 8 for comparison with the values obtained for the other proteins tested. The dissociation constants for SPF/TAP for other tocopherols, PI and PC are also reported and C. Panagabko determined these constants.

3.7 Photoaffinity labels deprotection and binding to α-TTP

Photoaffinity analogues of α-tocopherol were prepared by substituting a photosensitive functional group (tetrafluoroazido benzylxyloxy) at the end of the modified alkyl chain [55]. The alkyl chain varies in length from a hexyl to a nonyl extension (See Fig. 7).

These molecular probes were synthesised by J. Lei to further investigate the orientation of ligand binding of α-Tocopherol Transport Protein. The photochemical attachment of a label to a target molecule can facilitate the identification of the specific residue(s) involved in the binding of a ligand and the orientation and positioning of the ligand, thus providing information as to the structural details of the ligand protein interaction. During the synthesis of the tetrafluoroazidoaryl photoaffinity labels the phenol group on the chromanol ring was protected as the tert-butyldimethylsilyl (TBDMS-) ether [55]. A deprotection reaction monitored by TLC plates was done for the C₆, C₈, C₉ tetrafluoroazidoaryl photoaffinity labels. The purity of the successfully deprotected labels was also checked by TLC and their identities confirmed by mass spectrometry. Preparation of the C₇ label required only silica column purification as opposed to the deprotection reaction done to the other labels, for subsequent identification by its mass spectra.

The C₇ label was the first label tested since the C₇ linker yields a photoaffinity label with a side chain of the same length as the phytol chain of α-tocopherol. Thus the C₇ label best preserves, at a first approximation, the structure of the natural ligand. An initial binding screen showed the binding capability of α-TTP and Sec 14p to the C₇ photoaffinity label compared to α-tocopherol affinity (See Table 12). Subsequently, competitive binding curves with all of the labels and α-TTP were performed, yielding dissociation constants ranging from 3 – 9 µM (See
Table 13). The lowest $K_d$ values corresponded to the $C_7$ and $C_8$ photoaffinity labels. It confirms the importance of the length of the phytol chain for the binding affinity of $\alpha$-TTP and suggests that there is a size window preferred by $\alpha$-TTP to accommodate the 13 carbon length of $\alpha$-tocopherol’s phytol tail.

<table>
<thead>
<tr>
<th>Cold Ligand (M)</th>
<th>Sec 14p (% bound)</th>
<th>$\alpha$-TTP (% bound)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\alpha$-tocol</td>
<td>$C_7$ label</td>
</tr>
<tr>
<td>1.8e-9</td>
<td>89.0</td>
<td></td>
</tr>
<tr>
<td>5.0e-8</td>
<td>80.0</td>
<td>83.0</td>
</tr>
<tr>
<td>1.5e-7</td>
<td>76.0</td>
<td>90.0</td>
</tr>
<tr>
<td>1.0e-6</td>
<td>63.0</td>
<td>89.0</td>
</tr>
<tr>
<td>5.0e-6</td>
<td>33.0</td>
<td>67.0</td>
</tr>
<tr>
<td>2.0e-5</td>
<td>16.0</td>
<td>33.0</td>
</tr>
</tbody>
</table>

Table 13. Dissociation constants of recombinant human $\alpha$-TTP for various photoaffinity ligands

<table>
<thead>
<tr>
<th>Photoaffinity ligand</th>
<th>Dissociation constants (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_6$ label</td>
<td>$9.42 \pm 4.72$</td>
</tr>
<tr>
<td>$C_7$ label</td>
<td>$3.11 \pm 0.75$</td>
</tr>
<tr>
<td>$C_8$ label</td>
<td>$4.10 \pm 1.40$</td>
</tr>
<tr>
<td>$C_9$ label</td>
<td>$4.51 \pm 2.25$</td>
</tr>
</tbody>
</table>

3.8 Data Analysis

The Prism Graphpad 4.0 program provides a variety of options for the analysis of binding experiments. Analysis of the binding data included considerations like "shallow competitive binding curves". If the label or unlabeled ligand competes for a single class of binding site the competitive binding curve will have a shape determined by the law of mass action [46]. A standard competitive binding curve that follows this law has a slope of the linear portion of the logarithmic plot of $-1.0$. Quantification of the steepness of the competitive binding curves obtained with the proteins tested gave $-1.0$ slopes; which indicated that Sec 14p followed the law of mass action with a single site for all the ligands tested. Fitting homologous competition data (one site) included checking if a reasonable concentration of tritiated ligand was used. Homologous competition experiments only leads to useful results when the concentration of the radioactive ligand is less than the $IC_{50}$. Sec 14p binding experiments gave an $IC_{50} \geq 10 \times$ the concentration of tritiated ligand. As such, the concentration of the radioactive ligand was lower
than it needed to be. Several experiments were conducted with higher concentrations of $[^3]H\alpha$-tocopherol but the quality of data was the same and the dissociation constants obtained were very similar (within 5%) of those produced when lower concentrations of $[^3]H\alpha$-tocopherol were used. As a result, assays were continued with lower $[^3]H\alpha$-tocopherol concentrations to allow more experiments to be performed from a single preparation of $[^3]H\alpha$-tocopherol from the stock TBDMS-tocopherol.

Next, the $K_d$ and $B_{\text{max}}$ were determined and unexpectedly the $B_{\text{max}}$ value was high (~twice the expected value; which was 1). Further analysis of the data for one and two site competition was done to determine if Sec 14p might bind two molecules of tocopherol at 'two different sites' to account for the high $B_{\text{max}}$. Results always indicated that the best-fit equation was for the one site competitive binding curve. Another possible explanation to this unexpected result was the double occupancy of one site as it was observed in the solved Sec 14p structure with two BOG molecules in the binding site. This $B_{\text{max}}$ issue was addressed and solved by the adjustment of the $B_{\text{max}}$ initial value in the user-defined quadratic equation for homologous competitive binding with ligand depletion when fitting our data to this model. The user defined equation from the Prism Graphpad 4.0 program requires defining initial values by entering rules that allows Prism to compute the initial values of $B_{\text{max}}$, non specific binding and log $K_d$ for any set of data. A well-defined top and bottom plateau of the log of % bound within each binding curve also helped to improve the consistency/accuracy of the results.

It was also observed that the counting efficiency of the tritiated samples due to quenching was variable for each experiment run at different days. Chemical quenching is a reduction in the scintillation intensity seen by the photomultiplier tubes of the scintillation counter, due to materials present in the solution that interfere with the processes leading to the production of light, these materials quench the light once it is produced. A re-calculated constant that was used to transform the bound fraction (cpm) helped to represent better the quenching factor for each assay. Homologous and heterologous competitive binding curves with ligand depletion (one site binding model) provided the best results for the analysis of the binding data. The best-fit values for the $K_d$ and $B_{\text{max}}$ were evaluated for each assay according to their standard error, 95% confidence interval and the R squared of the goodness of fit.

3.9 Important quality control procedures

The purity of the deprotected tritiated $\alpha$-tocopherol was assessed prior to conducting binding assays. The integrity of the protein was periodically monitored by SDS-PAGE and protein concentration was determined immediately before assays were started. Cold ligand stocks
were frequently monitored by 10:1 hexane:ethyl acetate TLC to check purity (Rf determination of \( \alpha \)-, \( \beta \)-, \( \gamma \)-, and \( \delta \)-tocopherols were between 0.2 – 0.45), especially for ligands most susceptible to decomposition i.e. tocopherols.
4. DISCUSSION

4.1 Cell line transformation and gene sequencing

Prokaryotic transcription requires the binding of RNA polymerase to a promoter [56]. The minimal requirement for an effective gene expression system is the presence of a strong and regulatable promoter sequence upstream from a cloned gene. A strong promoter is one that has a high affinity for RNA polymerase, with the consequence that the adjacent downstream region is highly (frequently) transcribed. A high level of continual expression of a cloned gene is often detrimental to the host cell because it diverts large proportion of metabolic activity towards the expressed protein, which may be toxic thus impairing essential host cell functions [56]. Therefore, it is desirable to control transcription in such a way that a cloned gene is expressed only at a specific stage in the host cell growth cycle and only for a specified duration. The low-copy number pQE31 expression vector provides high-level expression of 6xHis-tagged proteins in E. coli based on the T5 promoter transcription-translation system (See Fig.22). This strong promoter is recognized by the E. coli RNA polymerase [52]. The optimized promoter-operator element in pQE31 plasmid contains two-lac operator sequences [52], which increase lac repressor binding and ensure efficient repression of the powerful T5 promoter. Tight control at the transcriptional level is then ensured with the combination of the special “double operator” system and high levels of the lac repressor protein generated by the lac I gene (See Fig 22). This lac repressor gene may be carried in the host chromosomal DNA or placed in a second plasmid carried by the host strain.

Fig. 22. pQE31 vector with a T5 promoter and a double operator. Reproduced from Qiagen [52]
The SG13009 E. coli host strain contains the low-copy plasmid pREP4 that constitutively expresses the lac repressor protein, which regulates the T5 promoter contained in the cloned pQE31 plasmid. The arrangement of two plasmids in a host strain is done in a way that they maintain different numbers of copies per cell. Usually, the repressor gene is placed on a low-copy number plasmid that maintains about 1 to 8 copies per cell, and the promoter sequence is inserted into a high-copy number plasmid that maintains about 30 to 100 copies per cell [56]. The low copy number plasmids (pQE31 and pREP4) in our expression system suggest that there was not an appropriate ratio between the repressor protein and the promoter. The effectiveness of deactivating a repressor protein and thereby activating transcription depends on the ratio of the number of repressor protein molecules to the number of copies of the promoter sequence [56]. Too many or too few repressor protein molecules would cause difficulties in inducing transcription or “leaky” baseline transcription at all times. Obviously, for the cloned Sec 14p gene this is not the case; the pREP4 plasmid would produce almost the same number of repressor protein molecules as the promoter sequences from pQE31. This indicates that the system used for the expression of the recombinant Sec 14p protein is effective and reliable. This was observed and confirmed in our experiments by the quantifiable amount of protein (0.5 – 1.0 mg/150 mL culture) obtained during the length of this research.

On the other hand, low plasmid DNA yields were obtained during SG13009 DNA extraction for plasmid insertion confirmation and sequencing purposes. Although this may be expected since the two plasmids (pQE31 and pREP4) from the QIAexpress system (SG 13009 E. coli strain) used for the expression of the recombinant Sec 14p protein are low-copy number. Large number of mini-preparations and even one or two macro-preparations were needed to isolate sufficient DNA for restriction analysis and sequencing. At the time, poor recovery of the pQE31/Sec 14p construct was attributed to the possible additional presence of the pREP4 plasmid from the initial preparation sent to us and the multiple copies inherent in the SG13009 cell line. Possibly a combination of both the low copy number of the pQE31 plasmid and the initial overabundance of pREP4 were responsible for this problem.

Plasmid DNA extraction was also done with the transformed JM105 E. coli host strain. The DNA yield from this cell line was more appreciable and readily visible on the agarose gels than for samples prepared from SG13009 cells. The JM105 host strain genotype indicates that this cell line carries a mutant Lac repressor protein as a single gene in the chromosomal DNA [57]. A mutant form of the lac I gene (lac I°) produces much higher levels of the lac repressor thereby decreasing the leakiness under noninduced conditions. JM105 may produce enough lac repressor to block transcription, but they would be less efficient than a strain that harbours the
pREP4 plasmid (SG13009), which produced more protein. The strong T5 promoter with a double lac operator needs a tighter regulation. JM105 genotype also features the endonuclease-A mutation that improves yield and quality of isolated plasmid DNA [57]. SG13009 does not possess this phenotype and may also explain the lower yield of DNA obtained. In summary JM105 was ideal for propagating the pQE31/Sec 14p plasmid but not as good an alternative for gene expression. The SG13009 *E. coli* host strain was the better choice for the expression of the fusion protein Sec 14p.

4.2 Protein Expression and purification

Expression of recombinant *S. cerevisiae* Sec 14p protein capable of binding its native ligand PI and other ligands has been achieved. The pQE31 expression system provided Sec 14p protein with an N-terminal 6xHis affinity tag. The hexahistidine tag enabled the binding to metal chelating surfaces as Ni-NTA gels for purification purposes and did not appear to interfere with the structure or function of the Sec 14p purified fusion protein as demonstrated by the protein’s binding capability. At pH 8.0 the 6xHis tag is small, uncharged, and therefore does not generally affect secretion, compartmentalization, or folding of the fusion protein within the cell [52]. On the contrary a study done to analyze the effects of even small N-terminal or C-terminal extensions (i.e. histidine tags) showed that such modifications can affect the stability of the native three-dimensional structure of the protein [58]. The presence of trace impurities bands observed on a SDS-PAGE after metal affinity purification of the soluble cell fraction was a concern for the binding experiments (See Fig. 12). Non-specific binding of contaminating *E. coli* proteins presumably contained several proximal histidine residues in their structure since they were simultaneously retained in the column matrix and eluted with the Sec 14p fusion protein. Optimization of the purification protocols and binding assays of the different fractions obtained from the protein expression and basic purification protocol were done to address the possibility that protein impurities contributed to the Sec 14p binding affinity observed. Results from these trials indicated that none of these contaminants affected the measurement of the binding behaviour of the recombinant Sec 14p protein and absolute protein purity is not required for the assays (See Table 4).

4.3 Binding Assays

The validity of the binding assays and the obtained results can be emphasized by the following general issues; the degree of uncertainty on the determination of low amounts of protein for Protocol 2 binding assays (Pg. 34) using the two methods available (Bradford and estimated Molar ExtinctionCoefficient) was compared with the amount of protein calculated
from the Bmax (binding capacity) of the binding equilibrium data analysis. In most cases these two results were close (~ 10 % difference), this internal check provided some assurance to the acceptability of these protein concentration determination methods. An estimation of the amount of protein being used for each assay was also calculated from the specific activity of the tritiated tocopherol and its amount bound (See Table 14 for a sample calculation). The proportion of the number of moles of protein used to the number of moles of bound ligand provided an indication of the amount of protein which was compared to the amounts determined by the methods available.

<table>
<thead>
<tr>
<th>Table 14. A sample calculation of the proportion protein:ligand for binding assays</th>
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<tr>
<td>For a 37000 MW protein and 0.4 µg used 0.4 µg/37000 ~ 11pmol of protein if volume of assay measured = (250/320 µL) = .78 11 pmol * 0.78 = 8.4 pmol of protein/assay</td>
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Practical evidence has indicated that kinetic experiments are more complicated and less accurate than equilibrium ones [45]. Even though preliminary kinetic studies indicated that binding equilibrium was achieved in an hour (See Fig. 23), it was decided to double the time to assure the establishment of true equilibrium.

Binding assays are based on the separation of the free from the protein-bound hydrophobic ligand by means of Lipidex gel. Lipidex matrix (an alkloxyalkyl derivatized Sepharose) has a high affinity for hydrophobic compounds and very low affinity for protein. This requires quantitative manipulation of hydrophobic ligands in aqueous solution, which confronts the tendency of these hydrophobic molecules to adhere to glass and plastic surfaces [51]. This tendency is overcome by the addition of detergents. In our research we found that some ³[H]-α-tocopherol was getting lost on the walls of Eppendorf tubes. A concentration of 100 µM Triton X100 detergent added to the incubation buffer helped to solubilize the hydrophobic ligands in buffer. Fresh solutions behaved as a stable aqueous dispersion and were used right away. Higher detergent concentration (200 µM) showed a 3 fold increased affinity of recombinant Sec 14p protein to α-tocopherol, likely the result of detergent stripping ligand from the lipidex faster and making it to appear in the bound fraction. The lipidex matrix showed sufficient affinity for the different ligands tested and allowed separation of the protein-bound and free ligand with comparable efficiency. When an entire
aliquot of any ligand tested was passed through the lipidex column without protein, > 90% of the ligand was trapped by the gel.

**Fig. 23. Time course study to establish the time needed to achieve equilibrium in the binding assays**

The effect of ethanol in ligand binding was studied by Schroeder *et al* [59]. Recombinant liver fatty acid binding protein (L-FABP) and sterol carrier protein-2 (SCP-2) were used to examine the effects of ethanol in vitro in ligand interaction. Ethanol at physiological concentrations (25 mM) dramatically inhibited NBD-stearic acid binding to SCP-2 [59]. Stock solutions of [3H]-α-tocopherol in ethanol were always added straight to the incubation solution in our binding assays. To evaluate the previously reported findings, binding assays were done with > 1% ethanol in the final assay. Assays with 1.6 to 2.2% final ethanol content produced the same degree of binding (~70%) than assays with lower ethanol concentration. The higher amount of ethanol in the assay did not cause an observable effect on the binding of α-tocopherol to Sec 14p. In spite of this, we always maintained a concentration of ethanol in the final assay below 1% (equivalent to 19 mM).

The independent determination of the protein concentration needed for binding experiments was established as a unique parameter that varied according to the characteristic affinity of each protein. For example 0.2 µg of the recombinant human α-TTP, 0.4 – 0.8 µg of the recombinant *S. cerevisiae* Sec 14p and 5 – 10 µg of the recombinant human SPF/TAP were needed to show
appreciable binding behaviour (> 50 %) under our chosen conditions of [\(^1\)H]-\(\alpha\)-tocopherol concentrations.

The range of hot (labelled) and cold ligand concentrations to be used were important and impacted the results initially obtained (Protocol 1 binding assays). The use of the smallest feasible concentration of label is recommended to estimate lowest possible \(K_d\) values [45]. An increase of non-specific binding was observed at higher concentrations of unlabelled ligand in all the assays performed perhaps due to the dilution of the radioligand that spread more to non specific sites.

4.4 Ligands chemistry

Small bio-molecules fall into three basic categories: hydrophilic compounds (e.g. carbohydrates and inorganic ions), hydrophobic compounds (e.g. triglycerides and cholesterol esters) and amphipathic compounds (e.g. fatty acids, bile acids, phospholipids, and bilirubin). Most cellular lipids are amphipathic [1]. Amphipaths are molecules with detergent properties, containing both hydrophobic and hydrophilic domains. As such they tend to bind to membranes and to various soluble binding proteins and have only limited solubility in water [1]. The ligands tested in this research include both hydrophobic (tocopherols, retinoids, cholesterol and squalene) and amphipathic molecules (phospholipids, a fatty acid and n-\(\beta\)-Octyl-glucopyranoside – BOG a detergent). The assayed tocopherols were RRR-\(\alpha\)-tocopherol, \(\beta\)-, \(\gamma\)-, \(\delta\)-tocopherol, \(\alpha\)-tocotrienol, \(\alpha\)-tocopheryl acetate, \(\alpha\)-tocopheryl quinone and Trolox a truncated water soluble \(\alpha\)-tocopherol derivative. The phospholipids phosphatidylinositol (PI) and phosphatidylecholine (PC), the monosaturated fatty acid oleic acid and the retinoids \(9-cis\)-retinal, \(trans\)-retinol and retinoic acid were also examined.

The hydrophobic tetrafluoroazidoaryl photoaffinity ligands were also tested against \(\alpha\)-TTP to explore their binding ability and establish their further use in structural and membrane studies of this protein. The photochemical attachment of a label to a target molecule can facilitate the identification of the specific residue(s) involved in the binding of a ligand and the orientation and positioning of the ligand, thus providing information as to the structural details of the ligand protein interaction. Four photoaffinity analogues of \(\alpha\)-tocopherol were prepared by substituting a photosensitive functional group (tetrafluoroazido benzyloxy moiety) at the end of the modified phytol chain. The alkyl chain varies in length from a hexyl to a nonyl extension (See Fig. 7). The seven and eight carbons extension might be the best to use because of their close approximation to the structure of the natural ligand. These molecular probes were synthesized by J. Lei to further investigate the ligand binding mechanism of \(\alpha\)-TTP for \(\alpha\)-tocopherol [55].
4.5 Sec 14p ligand binding

The binding abilities of recombinant *S. cerevisiae* Sec 14p for the 16 different ligands mentioned above were assessed to address Sec 14p’s similarity to the α-tocopherol transfer protein (α-TTP) and other member of the CRAL-TRIO lipid binding protein family. Sec 14p appeared a suitable choice for a control in the study of tocopherol binding characteristics of α-TTP and SPF/TAP, based on its sequence similarity and that Sec 14p was the first protein from the CRAL-TRIO family with an available three dimensional structure. Even before discovering its affinity for α-tocopherol, the Sec 14p structure allowed computer modelling of the α-TTP sequence. Unexpectedly, Sec 14p showed reasonable affinity for α-tocopherol, which afforded an opportunity to compare the binding behaviour of several members of the CRAL-TRIO protein family (α-TTP, Sec 14p, SPF/TAP and CRALBP). Such a comparison offered the possibility to shed light on vitamin E trafficking and metabolism.

In order to maximally represent and resolve the ligand preferences of each protein, a diverse set of ligands was employed. The chemical and structural characteristics of these ligands are illustrated in Figure 24. Structurally, the majority of these compounds are joined in a head to tail arrangement with linear hydrocarbon chains and cyclic groups at one end. Chemically the tails are saturated/unsaturated substituted hydrocarbon chains and the head is a combination of saturated/unsaturated rings substituted with polar and non-polar groups. The affinity and specificity of Sec 14p to BOG, a non-ionic detergent, found in the crystallized structure of Sec 14p [32] suggests that the characteristics to be recognized by Sec 14p are related to the head-tail structural arrangement and the amphipathic nature of the ligand provided by the substitution of polar groups on the head domain (i.e. hydroxyl groups). This correlates well with the chemical structure (amphipathic nature) of Sec 14p cognate ligand: PI. The comparison of the structure of PI and BOG shows that BOG’s head group is chemically similar to the inositol (See Fig. 24). The possibility of six potential hydrogen bonds between Sec 14p structure and the head group of BOG was suggested when the structure was solved [40]. This indicates the importance of hydrogen bonding groups on the head group (i.e. hydroxyl) of the ligand and the presence of polar residues on the inside surface of the Sec 14p binding pocket. The ligand-binding cavity of Sec 14p was described as a large hydrophobic pocket with a lipid exchange loop in an open conformation. This helix loop (A10/T4) is suggested to play important roles in membrane attachment and ligand discharge/extraction. It was observed that in this large ligand-binding cavity the BOG’s headgroup was oriented inwards close to the residues K239 and E207 that characteristically form a salt bridge among them. The detergent acyl chains extended away from the interior of the
binding pocket aligning with the extremely hydrophobic upper region of the pocket. This information suggests that the binding cavity of this protein possesses two defined areas to accommodate amphipathic ligands. The hydrophilic (polar) interior with structural groups (i.e. E207 and K239) that bind ligands through hydrogen bonding and a hydrophobic upper region where the hydrophobic domain of an amphipathic ligand makes extensive van der Waals contact with the binding pocket.

Based on a comparison of the chemical structures and the observed binding affinities of Sec 14p to the ligands tested, the following insights can be drawn. Incomplete competition of squalene suggests the importance of the amphipathic nature and shape requirement of the ligand in order to be bound by Sec 14p. This correlates well with the lack of hydrophilic domain on the squalene chemical structure and the absence of the rectilinear head to tail shape observed on the ligands bound (See Fig. 24). Additionally, even though their amphipathic nature, oleic acid and cholesterol were not bound by Sec 14p, and showed incomplete competition. This corroborates the shape requirement for the binding mechanism. Oleic acid is a straight long lipophilic hydrocarbon chain with a polar carboxylic acid group at one end. Meanwhile cholesterol has a tetracyclic base structure with four fused rings and a relatively small hydrocarbon chain at one end (See Fig. 24), neither of these two compounds resembles the head-tail structure arrangement and then they are not bound by Sec 14p. Trolox, a short chain α-tocopherol analog, has a relatively hydrophilic head (ring) but does not possess a hydrophobic chain (See Fig. 24). This compound lacks one of the characteristics that Sec 14p recognizes for binding, and thus Trolox is not recognized. The two retinoids tested with Sec 14p also showed incomplete competition. Thus it would seem that the lack of a significant polar functional group makes these inferior ligands for Sec 14p. All of this reaffirms the structural and chemical features important for Sec 14p ligand binding.
Fig. 24. Chemical structure of ligands tested with Sec14p, alpha-TTP, SPF/TAP and CRALBP
When BOG was assayed in competition with \(^{3}\text{H}\)-\(\alpha\)-tocopherol, incomplete competition was also observed. The Sec 14p protein was crystallized in the presence of 1% BOG, which exceeds its critical micelle concentration [40]. This detergent concentration was required for crystal formation. The required presence of BOG in the crystal structure does not indicate that this molecule will compete since competition would require displacement of tocopherol, an obviously better recognized ligand. Sec 14p demonstrated different dissociation constants and selectivities for tocopherols than did TTP. Sec 14p binds \(\alpha\)-tocopherol (\(K_d = 373\) nM) approximately ten-fold better than \(\beta\)-, \(\gamma\)-, and \(\delta\)-tocopherols. These three tocols are equally poor ligands (\(K_d \sim 4\) \(\mu\)M) but they show a slightly better affinity than \(\alpha\)-tocotrienol (\(K_d \sim 4.7\) \(\mu\)M) this demonstrates Sec 14p’s preference for ligands with a free phenol due to the hydrogen bond interaction during binding. \(\alpha\)-Tocopheryl acetate and \(\alpha\)-tocopheryl quinone without a hydroxyl on the head group (chromanol ring) exhibit the lowest affinity for Sec 14p (\(K_d \sim 6.0\) \(\mu\)M). The ten times better affinity of Sec 14p for \(\alpha\)-tocopherol compared to \(\beta\)-, \(\gamma\)-, \(\delta\)-tocols must be due to the full substitution of methyl groups on the chromanol ring. This methylation pattern results in a loss of free energy of binding of about 1.0 and 1.5 kcal/mol for the absence of a single methyl group at C7 in \(\beta\)-tocopherol and C5 in \(\gamma\)-tocopherol respectively [31].

The unexpected binding affinity of Sec 14p for \(\alpha\)-tocopherol was similar to the one obtained for its native ligand PI (381 nM). An explanation for these unusual results is based on the different protocol used during the preparation of the phospholipid stock “solution” and the modification in binding assays. Phospholipids were prepared as small unilamellar vesicles through sonication of the buffer hydrated lipid film. Addition of phospholipid vesicles into the assays required additional correction as it appeared that ‘free’ tocopherol associated with the lipid structures resulting in poor retention on the Lipidex matrix. As a result, samples containing no protein exhibited a much higher percentage of \(^{3}\text{H}\)-\(\alpha\)-tocopherol in the flow through or “bound” fraction, unobserved with the other ligands tested. Thus in attempt to correct for tocopherol encapsulated in vesicles, it was decided to implement protein-free controls for each phospholipid concentration of the binding curve. The tritiated tocopherol that flowed through the Lipidex under these conditions was subtracted from that observed in the presence of protein. Since it is possible that protein free controls do not accurately represent the interactions of tocopherol with the vesicles alone when protein is present, this correction may be too severe. Consequently, the control correction could likely account for an under-representation of the tocopherol in the bound fraction for PI and PC and thus the dissociation constants of Sec 14p for PI and PC may be lower than observed. In spite of such possible under-estimation of the absolute affinities, Sec 14p
displayed a ~ 16-fold higher affinity for PI relative to that for PC. This is consistent with the 19-fold higher intermembrane transfer rate for PI over PC observed with Sec 14p in an early report [39]. The ~ 16-fold lower affinity for PC illustrates also the importance of the ligand structural features to be recognized by Sec 14p. Finally, the greater affinity of Sec 14p for tocopherol than for other lipophilic ligands such as oleic acid, squalene or trans-retinol implies that the ligand-binding site of Sec 14p manifests a true selectivity for this ligand.

Sec 14p's ability to bind RRR-α-tocopherol raised the possibility of the existence of a tocopherol metabolic pathway in yeast. α-Tocopherol in a concentration of 0.47 μg/mL was reported in yeast in a study of lipid peroxidation in S. cerevisiae cells with ethanol treatment [60]. This work relates tocopherol as part of the antioxidant mechanism of yeast cells and they demonstrated that the amount of α-tocopherol decreased during ethanol stress [60]. Additional evidence for tocopherol distribution or metabolism in yeast has not been explored introducing the possibility that Sec 14p could potentially transfer tocopherol between membranes along with PI and PC.

4.6 Comparing Sec 14p to α-TTP and CRALBP ligand binding

From the binding affinities summarized on Table 8 it is clearly evident that α-TTP's affinity for α-tocopherol surpasses the other two proteins (Kₐ = 25 nM as opposed to 373 nM for Sec 14p and 528 nM for CRALBP) and thus α-TTP is truly selective for α-tocopherol. Since CRALBP, like Sec 14p, also bound α-tocopherol with similar affinity, it further supports that sequence homology within the ligand-binding pocket of these members of the CRAL-TRIO family is sufficient to allow recognition of similar ligands. Ligand structural features like the presence of a head group with hydroxyl groups was a factor of selection for Sec 14p. The same can be ascribed to α-TTP but with α-TTP exhibiting a preference for non-polar groups and only one hydroxyl substituent as opposed to Sec 14p recognition of six hydroxyl groups on PI and BOG.

α-TTP shows the capability to differentiate between the four natural forms of tocopherol. Thus, the different extent and position of methylation of the chromanol ring system influences binding. Specifically, α-TTP demonstrates a significant preference for a fully methylated α-tocopherol (25 nM) followed by β- (124 nM) which is methylated at the C-5 position, γ- (266 nM) lacks methylation at the C-5 position and δ- (586 nM) with a single methyl group on the chroman ring. This ranking of binding affinities correlates well with the reported ability of these molecules to inhibit the in vitro, TTP-catalyzed α-tocopherol transfer between membranes [25],
the biological activities as determined from rat gestation-resorption assays [4] and the biokinetics of α-, γ-tocopherol [61]. Thus, α-TTP’s ligand specificity supports the suggestion that α-TTP is an important discriminating physiological factor for the specific retention of dietary α-tocopherol. These observations are strongly supported by the very recently elucidated three dimensional structure of α-TTP [35]. The surface of the binding site of TTP is mostly lined with hydrophobic amino acids side chains in which the deeply buried ligand is surrounded by many hydrophobic residues through van der Waals contacts. Characteristically, this explains TTP selectivity for the different tocopherols due to the absence of methyl groups on their structure, this happens through a reduction of surface availability for hydrophobic interactions and a decrease in the packing density.

The higher affinity and selectivity of α-TTP for tocopherols is based on the two discriminators on the chromanol ring, the methyl group substitution and the phenol group. The importance of the free phenol in α-tocopherol for α-TTP binding is demonstrated by the poor recognition of α-tocopheryl acetate, α-tocopheryl succinate, α-tocopheryl quinone and 6-O-carboxymethyl α-tocopherol. Interestingly, the TTP solved structure illustrates that water molecules play an important role to inter-connect the phenolic hydroxyl group in 4-position on the tocopherol structure to the one hydrophilic amino acid (Ser140) and the backbone carbonyls through hydrogen bonding. α-TTP experienced a greater diminution in the binding affinity for the 2S stereoisomer (Kd = 545 nM) relative to the RRR-isomer than for loss of the single C5 methyl group in γ-tocopherol. According to the solved three dimensional structure the position and geometry of the pyran half chair of the chromanol ring determines the relative positions of the substituents at the C2-stereocenter with the axial methyl group protruding into an indent of the cavity formed by various hydrophobic residues [35].

Variations in the phytol chain differ significantly and do not show a clear trend as was evident for Sec 14p. Removal of the phytol chain in Trolox results in a 40-fold decrease affinity from α-TTP. This suggests that the chromanol ring discriminator has more relevance than the phytol side chain allowing Trolox to bind, albeit with lower affinity than α-tocopherol. The unsaturated chain of α-tocotrienol reduced its affinity to α-TTP 8-fold compared to α-tocopherol, but α-tocotrienol remained a better ligand than α-tocopheryl acetate, γ-, or δ-tocopherol. α-TTP demonstrated a Kd of 1.41 μM for PI, similar to α-tocopheryl acetate and 50-fold weaker than the affinity observed with α-tocopherol suggesting that the interior pocket of α-TTP possesses more hydrophobic surfaces that do not interact well with the hydroxyl groups from PI. α-TTP binding to oleic acid showed the highest Kd = 7.2 μM indicating a weak recognition of a straight
hydrocarbon chain, and emphasizing the importance of the chroman ring. Cholesterol, squalene, BOG, trans-retinol and retinoic acid failed to compete for α-tocopherol on α-TTP, similar to the results obtained with Sec 14p. All the above comparisons provide a good indication that the binding cavity of α-TTP is also a relatively large hydrophobic pocket with a less polar interior more on the hydrophobic side and a less hydrophobic region where the acyl tails are accommodated with less strong van der Waals interactions.

The assessment of the binding affinity of the four photoaffinity ligands tested against the recombinant α-TTP showed that none of these ligands bind this protein with high affinity ($K_d = 3-9 \mu M$). However, these affinities may be sufficient for the use of some of these ligands in protein structural and membrane studies. From these probes the C$_7$ and C$_8$ linker gave the best $K_d$s, 3 and 4 $\mu M$ respectively, for them to be considered in further studies.

4.7 SPF/TAP ligand binding

The degree of sequence similarity between α-TTP, Sec 14p and SPF/TAP is approximately the same (30%) (See Table 1), however SPF shows the least specificity and affinity for α-tocopherol than the other proteins tested $K_d = 615 \text{nM}$ (See Table 8). This result is comparable with previously reported affinities ($K_d = 460 \text{nM}$ [22]) and brings into question any possible role of this protein in vitamin E metabolism. The recently solved three dimensional structure of SPF can help to provide some explanations to the lower affinity showed by this protein for α-tocopherol and the different $K_d$s obtained with other lipid ligands tested. The reported SPF structure is described as exhibiting an N-terminal ligand-binding domain with a typical Sec 14p fold and a C-terminal of an 8-stranded jellyroll barrel [23]. Nonetheless it looks very similar to Sec 14p x-ray structure (See Fig. 25).
The binding site has a horseshoe shape with the entrance of the cavity blocked by the lipid exchanged loop constituting a ‘closed’ conformation. This conformation is further stabilized by the C-terminal helix of the jellyroll domain (See Fig. 25). The SPF structure solved represents the “carrier” state of the molecule where the resident lipid molecule is enclosed in the hydrophobic pocket. In order to open the pocket and to release the bound ligand into a membrane or other acceptor site, it is necessary to push the C-terminal helix away from the lipid exchange loop. The center of the pocket is occupied by 2 tyrosine side chains, which are hydrogen bonded to each other dividing the pocket in 2 branches. The pocket is mostly lined with hydrophobic amino acids side chains with the exception of a few amino acids that form salt bridges pointing towards the inside of the pocket. One branch of the pocket corresponds roughly to the location where the “inner” BOG1 molecule is found in Sec 14p and is occupied by an alkyl chain of > 16 carbon atoms, probably originating from the host cells (E. coli) used to produce the recombinant protein. Exchange experiments done with electrospray mass spectrometry, showed the complete exchange of the unidentified ligand by an excess of BOG, as well as PI. However, the binding stoichiometries (4:1 unknown, 4:1 BOG, 2:1 PI) observed from the mass peak ratios of apo-TAP/SPF versus the ligand complexes question the natural ligand binding site within the cavity of SPF. The reported displacement of the alkyl chain from the SPF crystallized structure by BOG...
and PI and not by tocopherol or squalene, correlates with the selectivity showed by SPF for PI which was the best ligand tested for SPF K_d (216 nM) (See Table 8).

SPF's affinity for γ-tocopherol with a K_d = 268 nM was comparable to that of PI, followed by β-tocopherol with a K_d = 393 nM. SPF indicated a greater affinity for the oxidized product of α-tocopherol (K_d = 441 nM) than for the α-tocopherol itself (K_d = 615 nM). δ-Tocopherol (K_d = 731 nM), squalene (K_d = 879 nM) and phosphatidylcholine (PC) (K_d = 1.2 μM) were the worse ligands in the specificity sequence showed by this protein with K_d's close to the μM range. Incomplete competition of oleic acid was also observed and a similar result was obtained with Sec 14p.

It thus seems that there are some parallels between the structural and chemical characteristics of the Sec 14p ligand binding and that of SPF with regard to the contribution of ligand polarity to the observed binding affinity of SPF. Even though the inner pocket of SPF is mostly lined with hydrophobic amino acids, the presence of hydrogen bonding and electrostatic interactions (salt bridges) could explain SPF affinity for PI. Similar to the proposed binding of PI to Sec 14p the polar groups of the PI head group could interact with the salt bridges stabilized through hydrogen bonding in the pocket. Such orientation could facilitate placement of the long fatty acids chains in the closed conformation of the SPF pocket. Meanwhile, oleic acid is not recognized by SPF possibly because it only has one polar carboxylic group at one end and a long lipophilic hydrocarbon chain that cannot provide sufficient contacts for polar interactions. This does not agree with the reported presence of the alkyl chain (> 16 carbon atoms) in the SPF structure. This disparity between the results obtained in this research and the solved three-dimensional structure makes it difficult to correlate them.

It is impossible to predict the native ligand of SPF based solely on the affinities of the ligands tested because they do not show a logical order and lack a clear description of ligand selectivity. One factor, which could explain the absence of an obvious trend in ligand binding, is the possibility that SPF requires additional regulation or activation for representation of 'true' ligand binding. The presence of regulatory mechanisms was explored in this research for α-tocopherol binding through the addition of modulators such as guanidine triphosphate (GTP), guanidine diphosphate (GDP) and divalent cations (calcium and magnesium) however, none of these conditions improved the binding affinity of SPF/TAP. Enhancement of SPF's ability to stimulate squalene monooxygenase was reported recently due to phosphorylation of SPF by ATP and protein kinases A & Cδ successfully [38]. Thus testing the K_d of SPF for α-tocopherol in the phosphorylated state is an important experiment that has already began in our laboratory.
The possibility that the location of fusion sequences in the recombinant protein perturbed the order or magnitude of ligand affinities was also explored. A number of recombinant proteins with different tag length and location were tested. The proteins with N-terminal tag were not able to specifically bind even at the highest amount of protein. On the other hand proteins with a C-terminal tag showed a defined affinity. This preference observed for SPF activity correlates well with the reported location of the binding site on the N-terminal of the solved structure since the N-terminal would be expected to be more sensitive to any disturbance caused by the fusion tags for ligand binding. In general it can be proposed that the complexity of SPF/TAP binding cavity could be related to contributions from its closed conformation and the division of the pocket in two branches. If one assumes that the same ligand shape (head and tail) is also recognized by SPF/TAP as the other proteins, it is difficult to predict where exactly the ligand head and tail would be positioned in the confined pocket. This was not a problem with the large open binding site of Sec 14p, which is able to accommodate ligands of larger size, such as PI, because of its almost rectilinear shape. Neither of the two independently ascribed abilities of SPF; its capacity to participate in tocopherol metabolism or to enhance squalene epoxidase, can be confirmed by our results. Thus, the in vivo ligand specificity of SPF and its physiological role still remain unclear.
5. CONCLUSIONS

5.1 The CRAL-TRIO proteins

Experimental observations in this research indicates that α-TTP, Sec 14p, SPF/TAP and CRALBP have overlapping binding specificities allowing recognition of common ligands. Taken together with the structural similarity in the binding pocket of this family of proteins, it suggests that this class of lipid binding proteins adopts a common fold in the CRAL-TRIO domain for accommodating their respective native ligand. However, each protein exhibited different degrees of recognition for a single ligand indicating the specialized feature in each binding region that makes molecular interactions more selective and unique for each protein. These specialized characteristics are the amino acids side chains that cover the inside surface of the pocket and provide for specific physicochemical interactions. Specificity of ligand binding is determined by the array of peptide backbone and amino acid side chains that decorate the ligand-binding cavity. As an example it has been reported that a single arginine residue can modulate ligand specificity and affinity in the Cellular Retinoid Binding Protein (CRBP) and intestinal Fatty Acid Binding Protein (FABP) [42]. The binding assays in this research suggests that the topological properties of the binding cavities of the proteins tested are mainly hydrophobic with some characteristic polar residues, this is in agreement with the solved three dimensional structure of Sec 14p, SPF and α-TTP.

All of the proteins in this study have long been assigned to the CRAL-TRIO family based on sequence similarity. Furthermore, the common structure that is assumed to exist between them supports the binding of similar ligands, albeit with different affinities and overall selectivities. At this point, it is also important to recognize from this work that simple binding of a lipid ligand does not necessarily imply that the binding protein functions in the biological processing of that ligand. The affinity, selectivity, specificity and stoichiometry showed by the protein are important indicators of the metabolic function of a given protein-ligand interaction. The CRAL-TRIO proteins showed differences in specificity and binding affinity for the ligands tested yet all appeared to have a ligand:protein stoichiometry of 1:1. An estimation of the ratio of protein to ligand from the specific activity of the tritiated tocopherol and its amount bound provided an indication of the proportion of moles of protein used to the number of moles of ligand.

5.2 α-Tocopherol Transfer Protein (α-TTP) ligand binding site

α-TTP clearly is best adapted to specifically recognize RRR-α-tocopherol because this ligand showed the lowest K_d (25 nM) and the highest affinity, strongly suggesting that of all
tested CRAL-TRIO proteins only α-TTP likely serves a physiological role in RRR-α-tocopherol metabolism.

The α-TTP binding pocket has considerable hydrophobic character that exhibits broad but distinct ligand specificity. α-TTP may facilitate more interactions with its ligand to achieve a higher binding specificity and many of these contacts involve hydrophobic amino acid residues. These observations are supported by the recently reported three-dimensional structure of α-TTP by Meier et al [35]. These authors suggest that α-TTP's specificity is governed by the packing density within the lipid binding pocket. The absence or presence of methyl groups on the chromanol head of tocopherols causes an increase or reduction of surface availability for hydrophobic ligand interactions thus increasing or diminishing the packing density. The different extent and position of methylation of the chromanol ring system of the tocopherol structure influences it's binding to TTP. TTP demonstrates a significant preference for the fully methylated tocol (α-tocopherol – 25 nM Kd) followed by the other two forms that lack one methylated group (β-, γ-tocopherols – 124, 266 nM respectively). δ-Tocopherol with a single methyl group on the chromanol ring showed the lowest affinity with a Kd of 586 nM. An important point to make is that even though β-tocopherol has the same number of methyl groups substituted than γ-tocopherol TTP could still differentiate β- from γ-tocopherol presumably based on the positional difference of the methyl substituents between these two tocols.

Distinctly, all the previously stated observations are reinforced by the following trends seen from the nineteen lipid ligands tested with α-TTP: fourteen were bound including even oleic acid and Trolox. These ligands were not recognized by Sec 14p showing incomplete competition. Five incomplete competitions were observed with α-TTP compared to seven from Sec 14p out of 16 ligands tested. On the other hand α-TTP clearly showed selectivity and ranked affinities for the different tocopherols tested. This was not observed with Sec 14p. α-TTP is capable to some extent of differentiating the nature and number of the substituents on the chromanol ring (head) being able to recognize slight structural differences among four natural forms of tocopherol.

The importance of the phenol group is demonstrated by the poor recognition of ligands with changes on this group i.e. α-tocopheryl acetate (Kd = 1.6 μM) and α-tocopheryl quinone (Kd = 814 nM). This selectivity factor can be attributed to polar amino acids (one or two) conveniently positioned in the architecture of the binding pocket. This observed selectivity on the solved structure of α-TTP was related to a Ser 140 side chain and water molecules that help to connect the phenolic hydroxyl group with the backbone carbonyls of Val 182 and Leu 189 [35]. Stereospecificity was also observed in the binding of TTP to the 2R and 2S stereoisomers of
tocopherol, very far apart $K_d$ values were obtained 25 nM for 2R and 545 nM for 2S. In conclusion the fine distinctions that $\alpha$-TTP recognizes from its cognate ligand (degree and position of methylation, hydroxyl group and the stereoisomer conformation) strongly supports the suggestion that $\alpha$-TTP is an important discriminating physiological factor for the specific retention of dietary $\alpha$-tocopherol. Even given these apparently specificities for $\alpha$-tocopherol, it would be wrong to conclude that $\beta$, $\gamma$- or $\delta$-tocopherol are unimportant physiologically because they may also have distinct, but as yet unknown, functions. For example the reported pharmacological functions of the $\gamma$-tocopherol metabolite $\gamma$-CEHC as a natriuretic factor [15] and the possible role of $\gamma$-tocopherol in titrating peroxynitrite [13].

The high binding affinity of $\alpha$-TTP to the photoaffinity ligands was disappointing and the ability to do further structure studies with some of them is questioned. Although, the $C_7$ and $C_8$ labels, which provided $K_{\text{obs}}$ of 3 and 4 $\mu$M could be good candidates for structural and membrane protein studies.

5.3 *S. cerevisiae* Sec 14p ligand binding site

The Sec 14p’s greater affinity for $\alpha$-tocopherol over the other lipophilic ligands such as oleic acid, squalene or *trans*-retinol implies that the ligand binding site of Sec 14p manifest a real selectivity for this ligand.

The Sec 14p lipid binding profile shows that the binding pocket of this protein has two well-defined binding domains that are equally important discriminators for the recognition of its ligands. The presence of electrostatic interactions in the pocket because of the presence of a salt bridge between the E207 and K239 side chains were described as important by Sha *et al* [32] for the formation of the hydrogen bond between Sec 14p and the headgroup of the ligand. Evidently, this is a suitable discriminator for Sec 14p’s specific recognition of the inositol moiety, which has six hydroxyl substituents versus PC, which has none. This polarity or presence of electrostatic interactions can not be ascribed to TTP’s binding site because of the observed ligand binding profile. The importance of the acyl chains or tails of the ligand for recognition by Sec 14p is well defined in our results and can be explained by the hydrophobic upper region of the Sec 14p pocket. Sec 14p did not bind Trolox, an analog of tocopherol with a fairly polar head and no tail. This illustrated and emphasized the importance of each of the two domains in a ligand; polar and hydrophobic, in order to interact with the polar and hydrophobic sites of the protein.

The solved three-dimensional structure of Sec 14p provided us a good model for the interpretative modeling of the $\alpha$-TTP CRAL-TRIO domain that is similar to Sec 14p. Sec 14p large pocket showed that tocopherol molecules could occupy this pocket, and in fact they do, this
was recently demonstrated by the crystallized structure of human α-TTP solved by Meier et al [35]. The molecular details of α-TTP binding to its physiological ligand were described in a closed “carrier” conformation in which the enclosed ligand is positioned differently from where our model initially predicted.

Sec 14p displayed the reported preference for its native ligand PC compared to PI confirming the activity and proper folding of the protein. In conclusion Sec 14p as an important member of the CRAL-TRIO family provided good evidence for the possible structural homology in the binding region between these proteins α-TTP and Sec 14p.

5.4 Supernatant Protein Factor /Tocopherol Associated Protein (SPF/TAP) ligand binding site

SPF/TAP showed the lowest affinity for α-tocopherol. Based on its binding characteristics it remains difficult to assess whether this protein should be ascribed a role in tocopherol metabolism. From the crystal structure the binding pocket of this protein is relatively large in its open state yet becomes obstructed by two helices in its closed conformation. The observation of alternate conformational states suggests the need of activators or modulators in order to open or close the pocket and thus regulate ligand binding. GTP, GDP, calcium and magnesium modulators did not improve the binding capabilities of SPF/TAP. Phosphorylation of SPF/TAP by ATP and kinases [38] enhanced its ability to stimulate squalene monooxygenase suggesting that phosphorylation may also improve α-tocopherol binding by SPF/TAP. This protein showed its highest affinity for PI from the ligands tested suggesting that SPF/TAP may bind phospholipids in vivo. The amino acids residues that line the inside pocket of SPF/TAP are mostly hydrophobic. They are buried by a hydrogen bond (between two tyrosine side chains) at the center of the pocket and salt bridges (between Glu127 and Lys124) at the entrance of the cavity making ligand entry difficult without conversion to the open state. Characteristically the presence of distinctive salt bridges is noted in the solved structures of Sec 14p and SPF, which could be related to their binding abilities.

Aside from these potential polar interactions it is important to mention that SPF was the only one from the proteins tested that showed recognition for the complex hydrophobic molecule squalene. The affinity demonstrated by SPF/TAP for this ligand was low at 879 nM showing that squalene is only moderately recognized but squalene binding to SPF/TAP could suggest that SPF/TAP’s binding to ligands requires hydrophobic interactions. Larger amounts of SPF/TAP where needed to achieve equilibrium and obtain good results and this could be interpreted also as higher binding stoichiometries. All this is well reflected in its reduced affinity to the ligands.
tested (i.e. α-tocopherol). On the contrary SPF/TAP showed fairly decent affinity to the oxidized product of α-tocopherol with a stoichiometry of 1:1.

Recombinant SPF/TAP proteins with a His-Tag located at the N-terminal did not show any appreciable magnitude of ligand affinity even at large amounts of protein used. Only an increase in non-specific binding was observed. The reported SPF/TAP structure [23] indicates that the binding region is located at the half of the N-terminal of the three-dimensional structure suggesting that the addition of fusion sequences might have disturbed the binding capability of the protein.

The lack of clear ligand selectivity by SPF/TAP to the ligands tested does not allow us to designate a true in vivo ligand for this protein. Thus its functional characterization continues to be an enigma at the present and requires further research.
6. FUTURE RESEARCH

The scarce knowledge on the molecular mechanisms of absorption, distribution and metabolism of vitamin E (RRR-α-tocopherol) makes it difficult to interpret its role in the etiology of diseases (i.e. atherosclerosis), and may underestimate its function and minimum dietary requirements, which are presently based only on animal models.

The determination of the absolute binding affinities of the recombinant human α-TTP and SPF/TAP for α-tocopherol and related ligands was a first step in the study of the role of proteins that specifically bind and potentially guide α-tocopherol to cellular and subcellular destinations. α-TTP’s high binding specificity for α-tocopherol is a good indicator of the physiological importance of this protein-ligand pair. No one has reported on the mechanisms of α-TTP assisted tocopherol transfer to and from membranes and, further research is currently required to achieve a molecular understanding of TTP’s specificity for tocopherol. An additional comparison of how the transfer activity and specificity of α-TTP varies between species would provide an insight into the effect of differences in protein sequence have on specific vitamer binding and transfer efficiency. Hydrophobic ligands can spontaneously transfer from one membrane to another but most likely they need the assistance of binding/transfer proteins that move them through the aqueous environment between membranes. The mechanism of this transfer may be diffusional, where TTP simply provides a “soluble” pool of tocopherol that allows intermembrane transfer, or it may require collision of the TTP with membrane surfaces during the pick-up and delivery of tocopherol. This later mechanism has been hinted at for TTP with the recent report of the structure of TTP [35]. The determination of the physicochemical parameters (i.e. concentration, composition and curvature of membrane and ionic strength of the medium) that affect the rate of protein-mediated transfer to membranes provides an indication of the mechanism involved either collisional or diffusion based. The determination of the mechanism by which tocopherol is transferred by its protein will thus help provide greater insight on the function and metabolism of this micronutrient. To this end, creation of a less laborious means for assessing tocopherol transfer and ligand binding would prove useful.

After preparation of acceptor membranes, small and large unilamellar vesicles (SUV or LUV) also bearing a fluorophore (phospholipid derivative), an in vitro transfer assay could be performed with a Fluorescence Resonance Energy Transfer (FRET) method for a direct observation of the change from a state of high fluorescence quantum yield for the tocopherol analog (free ligand) to a suppressed one (membrane bound). These questions and approaches
are currently being pursued in our laboratory and will likely contribute greatly to understanding TTP function.

Fig. 26. Structure of one of the proposed anthroloxy tocopherol 12-(9-anthroloxy)-2-dodecyl-2,5,7,8-tetramethylchroinaii

Reported studies describing the distribution of α-tocopherol in various subcellular compartments of the liver indicate the greatest concentrations of α-tocopherol were found in the Golgi membranes and lysosomes [62]. A cell based work of tocopherol transfer in vivo could be another useful approach to study the protein mediated transfer of tocopherol and to ascertain if ligand transfer by α-TTP is directed to very low density lipoproteins (VLDL) or other acceptor membranes and/or if other proteins are involved (i.e. Sec 14p like proteins in humans).

The demonstrated lack of specificity of SPF/TAP for tocopherol emphasized the need of a thorough investigation of the true physiological function of SPF/TAP and the search for its natural ligand. Only once the natural ligands are found, can better characterization of SPF/TAP’s ligand binding activity be assessed, possibly using a similar approach to the α-TTP studies through binding and transfer assays.

In regards to the elucidated three-dimensional structure of SPF/TAP, more research should be performed to determine the role of the jellyroll domain such as, whether interactions involving this domain have a role in targeting SPF/TAP to specific cellular sites. Studies can be outlined to demonstrate if this domain in this binding protein is surface active. A detailed structural analysis of the jellyroll domain could determine if it may contain electrostatic surfaces or amphipathic regions which may afford an affinity to membranes with different composition (i.e. higher proportions of anionic lipids). Amphipathic helices are known to be involved in protein membrane interaction via their ability to orient proteins at the polar/non polar interface of a membrane. The orientation of the amphipathic regions is also important to establish its accessibility to enter membranes or cellular sites. How SPF/TAP is activated (i.e. phosphorylation), the signal and the players involved, how many
ligands bind in the binding cavity, whether the effects caused by tocopherol on SPF/TAP's transcriptional activation [36] have any relevance, are all important questions.

Finally mutagenesis studies are also important tools on the functional investigations of these proteins. Mutant versions of α-TTP have been expressed and their binding tested, however, the recent announcement of the three dimensional structure of TTP will allow more specific structure/function questions to be addressed by mutation analysis. Mutations on the jellyroll domain of SPF/TAP could help to elucidate the interactions dictating the function of this domain.
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