Investigation of the Mechanism of Transfer of α-Tocopherol by the Human α-Tocopherol Transfer protein (h-α-TTP)

Grant E Frahm

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ABSTRACT

The human α-tocopherol transfer protein (h-α-TTP) is understood to be the entity responsible for the specific retention of α-tocopherol (α-toc) in human tissues over all other forms of vitamin E obtained from the diet. α-Tocopherol is the most biologically active form of vitamin E, and to date has been studied extensively with regard to its antioxidant properties and its role of terminating membrane lipid peroxidation chain reactions. However, information surrounding the distribution of α-tocopherol, specifically its delivery to intracellular membranes by α-TTP, is still unclear and the molecular factors influencing transfer remain elusive. To investigate the mechanism of ligand transfer by the h-α-TTP, a fluorescent analogue of α-toc has been used in the development of a fluorescence resonance energy transfer (FRET) assay.

(R)-2,5,7,8-tetramethyl-2-[9-(7-nitro-benzo[1,2,5]oxdiazol-4-ylamino)-nonyl]-chroman-6-ol (NBD-toc) has allowed for the development of the FRET-based ligand transfer assay. This ligand has been utilized in a series of experiments where changes were made to acceptor lipid membrane concentration and composition, as well as to the ionic strength and viscosity of the buffer medium. Such changes have yielded evidence supporting a collisional mechanism of ligand transfer by α-TTP, and have brought to light a new line of inquiry pertaining to the nature of the forces governing the collisional transfer interaction.

Through elucidation of the transfer mechanism type, a deeper understanding of the transfer event and the in vivo fate of α-tocopherol have been obtained. Furthermore, the results presented here allow for a deeper investigation of the forces controlling the collisional protein-membrane interaction and their effect on the transfer of α-toc to membranes. Future investigation in this direction will raise the possibility of a complete understanding of the molecular events surrounding the distribution of α-toc within the cell and to the body’s tissues.
null
ACKNOWLEDGEMENTS

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Finally, I would not have been able to undertake or complete this project without the total support of my family. The forms in which their help has come have indeed been diverse, and it would be impossible to quantify all that they have given over the years. Their interest, understanding and wisdom are beyond words, and serve as a reminder that one is never truly alone.

“...what a long, strange trip it's been...” - Robert Hunter
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<tr>
<td>α-toc</td>
<td>α-tocopherol</td>
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<tr>
<td>α-TTP</td>
<td>α-tocopherol transfer protein</td>
</tr>
<tr>
<td>AO-toc</td>
<td>anthracene-9-carboxylic acid 9-((R)-6-hydroxy-2,5,7,8-tetramethyl-chroman-2-yl]-nonyl ester (also, AO-tocopherol)</td>
</tr>
<tr>
<td>AVED</td>
<td>ataxia with vitamin E deficiency</td>
</tr>
<tr>
<td>CM(s)</td>
<td>chylomicron(s)</td>
</tr>
<tr>
<td>CMR(s)</td>
<td>chylomicron remnant(s)</td>
</tr>
<tr>
<td>CRALBP</td>
<td>cellular retinaldehyde binding protein</td>
</tr>
<tr>
<td>FABP</td>
<td>fatty acid binding protein</td>
</tr>
<tr>
<td>FRET</td>
<td>fluorescence resonance energy transfer</td>
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<tr>
<td>HDL</td>
<td>high-density lipoprotein</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl β-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>k</td>
<td>transfer rate constant</td>
</tr>
<tr>
<td>Kd</td>
<td>dissociation constant</td>
</tr>
<tr>
<td>Kn</td>
<td>partition coefficient</td>
</tr>
<tr>
<td>LBPA</td>
<td>2,2'-dioleoyl-lysobisphosphatidic acid</td>
</tr>
<tr>
<td>LDL</td>
<td>low-density lipoprotein</td>
</tr>
<tr>
<td>LUV</td>
<td>large unilamellar vesicle</td>
</tr>
<tr>
<td>MLV</td>
<td>multilamellar vesicle</td>
</tr>
<tr>
<td>NBD-toc</td>
<td>(R)-2,5,7,8-tetramethyl-2-[9-(7-nitro-benzo[1,2,5]oxdiazol-4-ylamino)-nonyl]-chroman-6-ol (also, NBD-tocopherol)</td>
</tr>
<tr>
<td>PC</td>
<td>phosphatidylcholine</td>
</tr>
<tr>
<td>PE</td>
<td>phosphatidylethanolamine</td>
</tr>
<tr>
<td>PI</td>
<td>phosphatidylinositol</td>
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<td>PS</td>
<td>phosphatidylserine</td>
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<td>PITP</td>
<td>phosphatidylinositol transfer protein</td>
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<td>PUFA</td>
<td>polyunsaturated fatty acid</td>
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<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate-polyacrylamide gel electrophoresis</td>
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<td>SET BUFFER</td>
<td>Sucrose-EDTA-Tris Buffer</td>
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<tr>
<td>SM</td>
<td>sphingomyelin</td>
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<tr>
<td>SUV</td>
<td>small unilamellar vesicle</td>
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<td>TRITC-DHPE</td>
<td>N-(6-tetramethylrhodaminethiocarbamoyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (also, TRITC-PE)</td>
</tr>
<tr>
<td>VLDL</td>
<td>very-low-density lipoprotein</td>
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1 INTRODUCTION

1.1 The Vitamin E Family

The name vitamin E describes a family of structurally similar compounds known as tocols, including tocopherols and their corresponding tocotrienols (T3's). The word tocopherol comes from the Greek words *tokos* (offspring) and *pherein* (to bear). Vitamin E was discovered as a necessary agent for the prevention of fetal re-absorption in female rats \(^1\), and early studies of its function emphasized its role as a lipid-soluble antioxidant that can maintain residency in cell membranes \(^2-4\). Specifically, vitamin E acts as a one-electron reductant (H• donor) in terminating the cycle of lipid peroxidation \(^2-5\), a function which imparts stability to cellular membranes by protecting unsaturated phospholipids from damage. The structures of the vitamin E family are comprised of a chromanol head group and a geranylgeranyl diphosphate-derived side chain, as shown in Figure 1.

![Figure 1: The structures of the vitamin E family members. Shown at right are the tocopherol (upper) and tocotrienol (lower) backbones, while chroman methyl group substituent configurations are listed at left.](image-url)
All tocots have a stereogenic centre at C2 and the tocopherols have additional stereocentres at C4' and C8', which are both biosynthesized in the R-configuration. The phytol tails are derived from geranylgeranyl phosphate and are either fully saturated for tocopherols or unsaturated at C3', C7' and C11' for tocotrienols. The extent of chromanol methylation yields α, β, γ and δ tocopherols and tocotrienols, for a total of eight naturally occurring vitamers.

The common structure of the tocots gives them generally similar physical properties and antioxidant potencies relative to other biomolecules. Even though these similarities exist, α-tocopherol is still selectively retained in tissues following ingestion and absorption, as evidenced by it being the most biologically active vitamer despite γ-tocopherol having the highest dietary abundance. This retention is reflected in typical plasma concentrations of ~27 µM and ~3 µM for α-tocopherol and γ-tocopherol, respectively. Although plants can synthesize α-tocopherol de novo (yielding d-α-tocopherol, which has RRR-stereochemistry), mammals must obtain α-tocopherol through dietary sources. Synthetic α-tocopherol, such as that found in supplements, is a racemic mixture, having an equal mixture of R and S configurations at the 2, 4' and 8' positions. This is referred to as all-rac α-tocopherol. Specificity by α-TTP for the 2R-stereoisomers for incorporation into the plasma is maintained, with (2R,4'R,8'R)-α-tocopherol exhibiting the highest tissue distribution. In fact, this stereoisomer normally accounts for ~90% of the vitamin E activity found in tissues.

1.2 Vitamin E As An Antioxidant

Though all members of the vitamin E family have similar antioxidant chemistries, the antioxidant mechanism and potency of α-tocopherol in particular have been extensively studied and debated due to selective retention of this vitamer in body tissues. The debate has been caused by contrasting evidence from a variety of techniques for assessing antioxidant potency. These experimental discrepancies are thought to be due to the presence of two different structural characteristics contributing to antioxidant efficiency. These include the degree of chroman methylation and the unsaturation of the side chain. Differential methylation of the chroman head group
is considered to be the major influence on chemical reactivity of tocols in donation of the phenolic hydrogen atom to terminate radical chain reactions \(^{20-25}\). Solution reaction rate constants supporting this general theory are as follows: \(\alpha\text{-toc} \approx \alpha\text{-T3} >> \beta\text{-toc} \approx \beta\text{-T3} > \gamma\text{-toc} \approx \gamma\text{-T3} >> \delta\text{-toc}\)
\(\approx \delta\text{-T3}\). A similar pattern is shown for tocols in small unilamellar vesicles (SUV), large unilamellar vesicles LUV and multilamellar vesicles (MLV) \(^{26}\), but some preferential consumption is shown for T3s compared to tocopherols, potentially due to increased mobility within the membrane \(^{8,20}\). Alternatively, the degree of unsaturation of the alkyl side chain is believed to affect the orientation of tocols in lipid environments, and thus their access to lipid radicals \(^{4,17,18,27,28}\). The mechanism of antioxidant action by \(\alpha\)-tocopherol is shown in Figure 2 \(^{29}\).

![Figure 2: The three general phases of the free radical chain mechanism of lipid peroxidation \(^{29}\).](image)

Damaging free radicals such as the superoxide radical \((O_2^•)\) are normally produced in cells, and can cause extensive damage to the lipid constituents of cell membranes. In
particular, the 1,4-pentadiene structures of polyunsaturated fatty acids (PUFAs) can come under attack from free radicals such as hydroxyl (HO•) and peroxyl (HOO•) radicals, resulting in the abstraction of a hydrogen atom (H•) from one of the methylene groups. The resulting carbon-centered radical (R•) is unstable and undergoes molecular rearrangement to form a conjugated diene, which in turn is attacked by molecular oxygen (O2) to form a peroxyl radical (ROO•). This peroxyl radical species is responsible for the propagation of the chain reaction by abstracting H• from a neighbouring PUFA, a process that could theoretically lead to complete oxidation of membrane PUFAs to hydroperoxides (ROOH) and is believed to result in physicochemical changes leading to membrane dysfunction. Such changes include alterations in membrane fluidity and oxidation of membrane proteins. α-Tocopherol donates a phenolic hydrogen to the fatty acyl (peroxyl) radical to stop this propagation and the subsequent attack on neighboring PUFAs. This results in the formation of an α-tocopheroxyl radical which is resonance stabilized by delocalization of the unpaired electrons at C6 into the aromatic ring of the chroman head. The α-tocopheroxyl radical can be reduced back to α-tocopherol by ascorbate (vitamin C), ubiquinone (Coenzyme Q10), or glutathione. Alternatively, it can dimerize with another α-tocopheroxyl radical or undergo further oxidation to form α-tocopheryl quinone. The relative ratio of [phospholipid]/[α-tocopherol] in the membrane environment ranges from 1000:1 to 3000:1, which places a high demand on α-tocopherol recycling mechanisms.

1.3 Vitamin E in the Lipid Bilayer

A full understanding of the protective function served by α-tocopherol is not possible without considering its orientation in the membrane and its interactions with the constituent lipids. The collective effects of the chroman ring, phytanyl side chain and hydroxyl group are known to determine α-tocopherol’s membrane orientation, as indicated by the study of tocopherol and tocopherol homologs. α-Tocopherol appears to be situated near the bilayer surface with the polar phenolic end ~5 to 7 nm below the water : lipid interface and the phytanyl tail directed down into the hydrophobic core, as in Figure 3. In fact, the chroman ring is believed to be located near the upper portion of neighbouring phospholipid hydrocarbon chains between
carbons 2 and 7 \(^{27}\). This orientation allows for access to radicals generated in both the lipid and aqueous phases\(^{17}\).

![Figure 3](image)

**Figure 3:** Positioning of \(\alpha\)-tocopherol within the lipid bilayer \(^{3}\).

Studies have suggested that \(\alpha\)-tocopherol in liquid-crystalline phase vesicles can lead to increases in acyl chain ordering, decreases in acyl chain mobility and interfacial polarity, but no change in surface charge \(^{36,37}\). The decrease in acyl chain freedom causes membranes to condense and lose both surface area and fluidity while the lipids assume a closer packing arrangement \(^{38}\).

\(\alpha\)-Tocopherol is believed to specifically induce negative bilayer curvature (similar to cholesterol) in comparison to \(\alpha\)-tocopherol hemi-succinate \(^{39}\). Phospholipids can have varying molecular shapes depending on the relative differences in the size of their head groups and hydrophobic tail regions (**Figure 4**), such that they will potentially pack into bilayers differently \(^{40}\).

![Figure 4](image)

**Figure 4:** Cartoon representation of the varying overall shapes of lipids including A) inverted cone, B) cylindrical and C) truncated cone. Shaded circles represent polar head groups which are connected to the hydrophobic tails. Adapted from Atkinson, Epand and Epand (in progress)\(^{40}\).
Lipids with relatively large head groups compared to their hydrophobic regions (inverted cone shape) are said to impart positive curvature stress to membranes they are deposited in, whereas lipids with smaller head groups relative to hydrophobic regions (truncated cone shape) impart negative curvature stress. Lastly, lipids with roughly similar head and tail regions (cylindrical shape) are said not to impart any curvature stress. The membrane packing stress resulting from the presence of certain lipids is thought to play a role in regulation of membrane-associated proteins.

Since most data describing α-tocopherol’s effects on membrane fluidity has been collected at concentrations of up to 30-40 mole %, the impact of α-tocopherol at physiological concentrations could actually be minor. With this in mind, however, it is impossible to accurately recreate the exceedingly complex cellular environment with respect to the presence and interaction of lipids, proteins and various solutes, so the complete in vivo effects of α-tocopherol on membranes may indeed be significant. This idea is substantiated by the fact that α-tocopherol treatment increased the rate-limiting step in aortic endothelial blood cell repair by altering the microviscosity of the cell membranes and increasing cell migration. Although some recent evidence of the membrane-stabilizing effects of α-tocopherol has been gathered at near-physiological concentrations in vitro, the total effect of the interaction of membrane constituents with α-tocopherol remains to be seen.

The described membrane orientation of α-tocopherol appears to be comparable to other lipids of similar structure with some exceptions. α-Tocopherol shows decreased mobility relative to plastoquinone due to stabilization of its complexes with phospholipids through hydrogen bonds between the C6 phenol and the phosphate oxygens or the carbonyl of the phospholipid head group. There is also evidence that α-tocopherol does not distribute uniformly throughout the bilayer. Techniques such as intrinsic fluorescence and x-ray diffraction have shown that α-tocopherol either exists in domains or preferentially associates with phospholipids with unsaturated fatty acyl chains.
1.4 Absorption and Processing of α-Tocopherol

Vitamin E utilization involves dietary absorption, transportation to cells and incorporation into cell membranes \(^{31,42}\), and since all vitamers in the tocol family are hydrophobic, there must also exist a special method of transport through the aqueous cellular medium. Once vitamin E is ingested, any natural tocols are absorbed into the lumen of the small intestine \(^{42}\) (Figure 5). The acetate and succinate esters (the common forms in supplemental vitamin E) are first hydrolyzed by non-specific lipases. Once absorbed into the enterocytes lining the gut, vitamin E is incorporated into chylomicrons (CMs) along with esterified fatty acids and cholesterol, and is secreted into the intestinal lymph (chyle) prior to entry into the plasma \(^{43,44}\). Next, CMs are partially catabolized by lipoprotein lipase (LPL), an endothelium-bound enzyme, yielding chylomicron remnants (CMRs). This process is concurrent with the distribution of some free fatty acids and tocopherols to extrahepatic tissues. The CMRs are transported to the liver and taken up via receptor-mediated endocytosis in the parenchymal cells \(^{31}\). Here, the CMR contents are processed and repackaged in very-low-density lipoproteins (VLDLs), into which \(RRR\)-α-tocopherol is specifically incorporated \(^{45-47}\). α-Tocopherol-containing VLDLs are secreted into the plasma by exocytosis from hepatocytes, while excess α-tocopherol and other isomers are secreted into the bile. Subsequent VLDL lipolysis by LPL to generate low-density lipoproteins (LDLs) results in α-tocopherol having three fates. It can be

![Figure 5: Pathway of vitamin E metabolism after ingestion \(^{42}\).](image-url)
transferred to HDL for delivery to tissues, remain in VLDL remnants and return to the liver, or remain in LDLs, which are the dominant vehicle by which α-tocopherol is delivered to peripheral body tissues.\(^{43, 48}\)

Enzymes such as cytochrome P450 4F2 (CYP4F2, located in the endoplasmic reticulum) degrade any tocols that are not retained (primarily 2S-α-tocopherols, tocotrienols and β-, γ- and δ-tocopherols) during the secretion process. Although CYP4F2 shows greater tocopherol-omega-hydroxylase activity for γ-tocopherol over α-tocopherol, the 7-fold serum enrichment of α-tocopherol over γ-tocopherol (despite a higher dietary intake of γ-tocopherol) suggests the presence of a more specific mechanism for the selective uptake of α-tocopherol.\(^{12, 49}\) This mechanism is now understood to be the specific binding of α-tocopherol by the α-tocopherol transfer protein (α-TTP) in the liver for incorporation into VLDLs.\(^{27, 50}\)

1.5 Hydrophobic Ligand Binding Proteins

1.5.1 The CRAL-TRIO Protein Superfamily

α-TTP is a member of the CRAL-TRIO protein family, which consists of 273 members ranging in source from mammals to lower eukaryotes, *Drosophila* and *Arabidopsis*.\(^{51-55}\) Two domains characterize homology within this family. The first is a short N-terminal 3-helix coil motif (the CRAL-TRIO domain) and the second is a β/α/β motif (the Sec14p binding domain).\(^{56}\) (Figure 6). These structural domains are so designated because of the proteins they are derived from, namely the human cellular retinaldehyde binding protein (CRALBP), the Dbl homology multi domain protein TRIO, and the yeast phosphatidylinositol (PI) transfer protein Sec14p.\(^{51-53, 57}\) Nomenclature is
variable for these regions, where one disparity, for example, has the Sec14p region described above sometimes being called the CRAL-TRIO domain. This is most likely due to the similar structures of all proteins in the family and the fact that different groups study different proteins and refer to their own protein as the “prototype” for structural motifs in the family.

CRALBP is a soluble monomeric 318 aa lipid binding protein that has, despite being expressed in a variety of locations, been studied primarily for its role in visual system tissues. Specifically, CRALBP has been characterized extensively in the retinal pigment epithelium and Müller cells, the sites of 11-cis-retinal synthesis. 11-cis-retinal is produced by regeneration from all-trans-retinal after photobleaching, and is the preferred ligand of CRALBP. CRALBP appears to play a key role in the visual cycle by directly binding 11-cis-retinal and 11-cis-retinol, and by influencing certain visual cycle enzymes. The proposed role of CRALBP is supported by CRALBP (-/-) mice exhibiting a 10-fold reduction in recovery after photobleaching, 11-cis-retinal synthesis and dark adaptation. It is now understood that mutations in the CRALBP gene can cause retinal dysfunction, manifested by a variety of conditions including retinitis pigmentosa. Of the six mutations contributing to these conditions, three are missense mutations (R150Q, M225K and R233W) which correspond to residues R107, V182 and K190 in α-TTP, as shown in Figure 7.
Human CRALBP: SEGVTFRMVPEEEQELRALELQTTDKHPVGPSCQLP HTLQAKDLENERETREE 60
Human α-TTP: MAEARSQPAGQPLNALDPSLQPAAL PRARAGVPLAPLRT--- 48
Yeast Sec14p: VQQEKEFELPSQCPFLAPDGPTKNDLSA EKALRELKLEEDAFIER 51
Human SPF: MSGRTGDLPFKKEALAKF ENVQVDPALPN----- 32

Human CRALBP: AVRELQEMVQAASGEELAVAAVEQKEDSIF LR RARKF GR YE RGYV R 120
Human α-TTP: DSF LR RADDF DL WR PKNY R 77
Yeast Sec14p: LDDST LR RARKF QL KE ENCE R 82
Human SPF: PDDYF LR RARSF QK EA PRHV R 6

Human CRALBP: L Y---PELFDS SPEAVRCT EAGYPGVLSRDKYGRVVMFLN ENW S------QEI FDE 174
Human α-TTP: A C------PEISAD HPRISLG KAGYGHVLSPRDoGSVYR AHM P------KVF AYD 131
Yeast Sec14p: K YGTDTILQD HYDEKP L AKFYPOYHTXDKGFPYFVYEE GAV LH0MKNV TEE 141
Human SPF: K K------DIDY ISWQPPEV QQVLSGMGDYLDGCCFNYDI GPL AGKLLPSA KQD 119

Human CRALBP: AYCFROLE N----------ETIQ GFCIIE P GFT QQAASLRTSDR MVD L 226
Human α-TTP: VSLITSEL E------VETOR GIKAI F L GWQ SHAFOITPSVAK IAA L 183
Yeast Sec14p: NLWWEYES YRLPACRAAGHLV TSCTIM L GIS SSAYSVMS-YVR ASY S 200
Human SPF: TKMREC EL ECAGQTT--KLGRKV ITIIY C GLG KHLWKPAEYAG FLC F 178

Human CRALBP: SD GGT------LPKY GKAUEQLFGPQAOAINTAF----- 316
Human α-TTP: LE GGE------EFSM DQCEWTNFIMKEDSYSLSSLSEQ-- 278
Yeast Sec14p: VK GKSBEVDK------GGLYLS IGPWDRPKYIGEEOAPEAFSMMK------ 303
Human SPF: VE GGMTDPDNGPKCKSKINYGGDIPKYVR QVKQQYHESVQISRGSSHQVEYEILF 298

Figure 7: Sequence alignment of four members of the CRAL-TRIO family, including human CRALBP, human α-TTP, yeast Sec14p and human supernatant protein factor (SPF). Regions of sequence homology between all four proteins are shown by coloured amino acids, where identical residues are pink and similar residues are green. Residues sharing similarity between 3 of the four proteins are coloured blue. Alignment was generated using the BLOSUM62 matrix default (taken from Panagabko, 2003).

TRIO is a member of both the CRAL-TRIO family and Dbl homology family of guanine nucleotide exchange factors. It has been implicated in the regulation of focal adhesion dynamics and also in the activation of Rho family GTPases in cytoskeletal remodeling.

Sec14p is a yeast protein that is involved in protein secretion and regulation of lipid synthesis and turnover in vivo, but acts as a PI-PC transfer protein in vitro. It is interesting to note that although Sec14p exhibits no similarity to mammalian PITPs, such as the retinal degeneration proteins PTPα and PTPβ, it can rescue PTPα (-/-) mutant mice.

Structural and biochemical evidence indicates that the ligand binding cavity is contained in the pre-described Sec14p domain (Figure 6 B), and that mutations within this domain are implicated in functional impairments of CRAL-TRIO family proteins.
Structural and biochemical evidence indicates that the ligand binding cavity is contained in the pre-described Sec14p domain (Figure 6 B), and that mutations within this domain are implicated in functional impairments of CRAL-TRIO family proteins. Structure/function studies of Sec14p have also suggested that ligand abstraction and deposition into membranes occur via a mobile lipid-exchange loop that can insert into the bilayer core after docking of the protein at the membrane. It is now known that the α-TTP shares similar sequence and structural motifs with the CRAL-TRIO family and also exhibits ligand-specific binding interactions with α-tocopherol, as other members do with their respective ligands. It is therefore also thought that the α-TTP might act in a similar manner to Sec14p regarding ligand pickup and delivery to membranes.

1.5.2 α-TTP

The human α-TTP (h-α-TTP) is a 278 amino acid protein with a molecular weight of approximately 32 kDa. It has been located in the mammalian brain, uterus, heart, spleen, kidney and liver, with liver showing the highest expression levels. α-TTP in the mammalian brain appears to be essential for protection against neuronal damage by oxidative mechanisms, while mouse uterine α-TTP is reportedly essential for embryogenesis by supplying RRR-α-tocopherol to the labyrinth region of the placenta during development.

Crystallography studies of the α-TTP show that most of the amino acids in the binding site are hydrophobic, and are situated to form a classical lipid binding cavity within the protein. Exceptions to this are Ser136, Ser140 as well as three water molecules, all of which are positioned around the phenolic hydroxyl group and serve to stabilize this group by hydrogen bonding. A small hydrophobic pocket is formed by Ile194, Val191, Ile154 and Leu183, into which the C5 methyl group fits snugly (as shown in Figure 8). Similarly, the C7 and C8 methyl groups are stabilized through
Figure 8: Schematic representation of the α-tocopherol binding site within α-TTP. Possible hydrogen bonds are indicated by broken lines and van der Waals contacts are shown with small arcs.

hydrophobic interactions with Phe187, Phe133 and Leu137, while the C2R methyl group rests in a pocket formed by residues Phe133, Val182 and Ile179. The side chain folds back on itself in a U-turn shape involving movement at the 4' and 8' stereocentres. Both in vivo and in vitro experiments using eight stereoisomers of α-tocopherol have shown that α-TTP is stereoselective toward R-configuration at the C2 position of the chromanol ring, where the protein exhibits a 10 - 20-fold lower affinity for synthetic SRR-α-tocopherol than for the natural RRR-diastereomer. This lower affinity is believed to be largely due to a loss of hydrophobic packing interactions at C2 with the corresponding residues described above, while fewer packing restraints and greater flexibility of the side chain result in the 4' and 8' positions having much less of an impact on binding.

Further analysis has shown the α-TTP to exist in one of two possible conformations (Figure 9). The first conformation (holo-α-TTP) is commonly referred to as the “carrier” conformation because the lipid exchange loop, or lid (residues 198-221) is in the closed position. The second conformation (apo-α-TTP), called the “membrane-docking” conformation, is so designated because the lipid exchange loop is...
in the open state, which is presumed to facilitate ligand migration into or out of the binding site, as well as a possible interaction with a lipid bilayer. 

**Figure 9:** Structure of the $\alpha$-TTP in both the A) open and B) closed conformations, in the absence and presence of $\alpha$-toc, respectively. The key residues of the mobile lipid exchange loop (198-221) are shown in red, while the key residues of the binding site which stabilize the phenolic ring of $\alpha$-toc are shown in green. $\alpha$-toc is shown in black as a stick diagram. Note that the open conformation is actually bound to Triton X-100 detergent (not shown).

Specifically, the closed conformation shows a large hydrophobic area of the lid (Phe203, Val206, Phe207, Ile210 and Leu214) as being in direct contact with the side chain of $RRR-\alpha$-toc. In the open conformation of $\alpha$-TTP, these residues shift toward the protein’s exterior where potential membrane interactions could occur simultaneously with ligand migration. Such a mechanism has been described for the non-homologous PTP$\alpha$ while similar conformational changes have been observed in Sec14, as well as lipases (which also deal with lipid-water interfaces). In all of the above cases, the lid comprises an $\alpha$-helical structure.

1.5.3 Ataxia with Vitamin E Deficiency

Ataxia with Vitamin E deficiency (AVED) is a rare disorder caused by mutation of the $\alpha$-TTP gene and subsequent failure to incorporate $\alpha$-toc into VLDL. The observed phenotype is characterized by cerebellar ataxia and general neurodegeneration resulting in loss of balance, memory and motor control. AVED, in the absence of
fat malabsorption, is unique to other forms of VED because these conditions result from the impaired synthesis of chylomicrons, VLDLs and lipoproteins, not specifically from disrupted transfer of α-toc due to α-TTP gene mutations \cite{88,90}.

The α-TTP gene mutations associated with AVED contribute to differing severities of the disease depending on their position and the resulting amino acid interactions \cite{62,75}. Missense mutations R192H and H101Q, where semi-conserved amino acids are replaced, are associated with mild (late-onset) AVED. R192 is located at the exit of a water-filled tunnel connecting the binding cavity with the bulk solvent and the R192H mutation results in the replacement of a hydrophilic arginine residue with a residue of intermediate polarity. Thus, neither protein function nor stability is dramatically altered \cite{75}. H101 is located beside helix 9 (residues 129-143), which is a building block of the CRAL-TRIO fold, forming one wall of the α-toc binding cavity. This residue participates in hydrogen bonding with T139, and the H101Q mutation does not completely destroy the interaction, leading to a mild disease phenotype \cite{75}.

The E141K missense mutation results in disruption of a hydrogen bond between E141 on helix 9 and Y73, resulting in severe AVED (most likely due to destabilization of the CRAL-TRIO fold) \cite{75}. R59 and R221 are located in a cluster of arginine residues on a surface patch that is highly positively charged (Figure 10).

**Figure 10:** Structure of α-TTP including the proposed membrane interaction surface. Protein is viewed with the binding site in the upper middle section, and hydrophobic (yellow), basic (blue), acidic (red) and polar residues (cyan) are shown, along with residues 198-221 of the hydrophobic lipid exchange loop (grey) \cite{75}.
Both the R59W and the R221W mutations lead to the replacement of surface positive charges with hydrophobic residues, while the R59W mutation also causes the loss of a salt bridge with D185. The introduction of a hydrophobic group on the protein's surface could lead to protein aggregation, while the loss of a salt bridge would most likely destabilize the overall protein structure.

It could also be hypothesized that positive charges on the protein surface may contribute to membrane docking by interaction with negatively charged lipid headgroups. Support for such a hypothesis is found in the fact that the K239A missense mutation in Sec14 completely abolishes phosphatidylinositol transfer activity in vitro (K239 is conserved in all Sec14 homologs and corresponds to R221 in α-TTP, Figure 7). Like R59 and R221 on α-TTP, K239 is also part of a cluster of positively charged residues on the Sec14 surface.

Many of the findings to date regarding AVED pathology have been relatively macroscopic in scale (i.e.- whole organism studies) and have come from in vivo studies. However a more detailed investigation of the impact of given mutations on the kinetics and mechanism of transfer of α-TTP has recently been undertaken.

1.6 Hydrophobic Ligand Transfer Studies
1.6.1 Ligand Interactions with Binding Proteins

Because the movement or diffusion of any free hydrophobic ligand through the aqueous cellular environment is thermodynamically unfavourable, there must exist some mediating factor to circumvent this problem. This mediating factor is realized in the form of hydrophobic ligand transport proteins, which help to overcome the thermodynamic barriers to cytosolic ligand diffusion while affording a degree of control to the transport process. Examples are seen in the cellular retinaldehyde binding proteins (CRALBP)58,97, phosphatidylinositol binding proteins (PITP)98, fatty acid binding proteins (FABP)99-107, sterol carrier protein (SCP)108-110, glycolipid transfer protein (GLTP)111, and in the α-tocopherol transfer protein (α-TTP)95,112-114.

To appreciate the role of transport proteins in the cytosolic flux of hydrophobic ligands, one must first understand the individual steps that comprise the overall transport process. Probably the most easily recognizable step is the simple one-site equilibrium
model for binding of ligand by receptor, which is described by Motulsky and Christopoulos\textsuperscript{115,116} according to the law of mass action as:

\[
\text{Receptor} + \text{Ligand} \xrightleftharpoons[k_{\text{off}}]{k_{\text{on}}} \text{Receptor} \cdot \text{Ligand}
\]

where \(k_{\text{on}}\) is the receptor-ligand association rate constant and \(k_{\text{off}}\) is the receptor-ligand dissociation rate constant. This equation can specifically be expressed in terms of the ligand-protein interaction as Cheng and Prusoff\textsuperscript{117} did, where \(L\) is the ligand, \(P\) is the protein and \(k_1\) and \(k_{-1}\) represent the rates of complex formation and dissociation, respectively\textsuperscript{117}. From this relationship, the degree of interaction between a protein and its ligand can be determined. This measurement is known as the dissociation constant (\(K_d\)) of the complex and is a comparison of the relative rates of formation and dissociation of the complex at equilibrium:

\[
K_d = \frac{k_{-1}}{k_1} = \frac{[LP]}{[L][P]}
\]

Essentially, the \(K_d\) value provides information in energetic terms on the ability of a protein to recognize and bind a ligand\textsuperscript{115,116}.

1.6.2 Hydrophobic Ligand Transport – A Mechanistic View

A simple description of the binding of ligand by protein, however, is not sufficient to completely illustrate the many events that occur during ligand transport to or between different target sites. Since these sites are typically either biological membranes\textsuperscript{101, 103, 105, 109, 118-120} or macromolecular complexes\textsuperscript{121} for which the hydrophobic ligand has some natural affinity, some consideration must be given to the analysis of ligand movement into and out of these domains. Moreover, consideration must also be given to the potential for recognition of target membranes and/or specific membrane domains by a transport protein\textsuperscript{100, 102-104, 122, 123}. This leads to the construction of a more inclusive and detailed model for ligand transport whose complexity varies with the number and type of
domains involved, as well as the extent of protein-membrane interaction. With this in mind, it is important to now consider the two general mechanisms of hydrophobic ligand transport.

Passive, or diffusional transfer occurs when a protein acquires and releases its ligand into the cytosol in the vicinity of a target membrane\textsuperscript{101, 105, 124}. Since there is no specific interaction between the protein and the target membrane, ligand transfer ultimately occurs by diffusion and the mechanism is designated as the diffusional mechanism of transfer. Here, the protein is acting as a carrier or helper, facilitating ligand solubility and migration through the bulk aqueous environment.

A variation of this mechanism involves the formation of ternary complexes between the transport protein and its target\textsuperscript{125}, with ligand being released in close proximity to the target membrane. The ligand, however, is still believed to ultimately diffuse through the aqueous cellular environment to its target.

When ligand pickup and/or release is contingent on a specific protein-membrane interaction, the transfer event increases in complexity, and is designated as collisional\textsuperscript{100-102, 122, 124, 126}. The increase in complexity comes from the requirement for protein recognition of the target membrane, adsorption to that surface, direct pickup or release of the ligand, and subsequent desorption of protein from the membrane.

A graphic of the two general types of protein-mediated intermembrane ligand transfer (Figure 11) shows one mechanism that includes a diffusional step and one which does not:

A) 

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig11.png}
\caption{Cartoon representation of A) Diffusional and B) Collisional ligand transfer. In the case of diffusional transfer, the ligand/protein complex dissociates, and free ligand migrates through the aqueous medium to the acceptor membrane. In the case of collisional transfer, ligand is deposited into acceptor membrane after the protein comes into contact with the membrane.}
\end{figure}

B)
A more detailed study of the diffusional transfer scheme shows that the ligand must migrate out of the donor membrane into the aqueous cytosol, where it binds to its corresponding transport protein. The protein facilitates movement of the ligand through the aqueous milieu and at some other point releases the ligand, which once more undergoes a thermodynamically unfavourable diffusion process until it deposits into the target, or acceptor, membrane \(^{101,103,105}\).

Alternatively, during what is known as collisional ligand transfer the protein has the potential to recognize and bind to the donor membrane, either through electrostatic or hydrophobic interactions \(^{100,101,103,109,122,127,128}\). Often, these interactions are concurrent with a conformational change of the protein to promote ligand migration \(^{62,75,100,129}\). The ligand is in this case "picked up" directly out of a membrane by the protein, and once protein desorption from the membrane occurs, the complex freely moves through the cytosol until the protein recognizes an acceptor membrane site. Upon recognition, the same interactions that facilitated ligand pick up can promote deposition of the ligand into the acceptor membrane bilayer. Following protein desorption, the protein is free to find another donor site.

The mechanisms by which different proteins transport their ligands are often elucidated by similar experimental protocols. The rate of diffusional ligand transfer is sensitive to changes in the ionic strength of the medium \(^{101,103,105,124}\) such that higher ionic strength effectively lowers the aqueous solubility of a hydrophobic ligand, so increasing the concentration of salt in the assay buffer will diminish the transfer rate. Diffusional transfer, however, is generally unaffected by increases in the acceptor lipid concentration or by changes in the composition of acceptor phospholipid bilayers (incorporating lipids with charged head groups, for example). This is because the rate-limiting step of diffusional transfer is the spontaneous movement of ligand out of the donor membrane \(^{103,105,124}\).

Although rates of collisional transfer can also be affected by changes in buffer ionic strength, it is thought to be for different reasons than those described for diffusional transfer. Collisional transfer can, in some cases, be diminished by increases in ionic strength due to disruption of electrostatic membrane-protein interactions \(^{104,105}\). Interestingly, some studies actually report slightly higher rates of collisional transfer
under conditions of increased ionic strength, but this is believed to be due to changes in either bilayer structure or overall protein conformation.

The main characteristics of collisional transfer that are most often exploited for mechanism identification are sensitivity to changes in acceptor lipid concentration and bilayer phospholipid composition. Since the protein binds to the donor and acceptor membranes and direct ligand migration occurs to or from the protein, an increase in concentration of acceptor will result in a corresponding increase in transfer rate due to the larger available surface area. Also, depending on the forces involved in protein recognition of target membranes, changing the phospholipid composition of these membranes can increase the rate of transfer. For instance, if a collisional transfer protein interacts with a target membrane through a cluster of basic amino acid residues, the addition of anionic phospholipids to the membrane will increase the protein’s affinity for it, resulting in a potentially higher rate of ligand pickup and/or delivery.

Although a variety of lipid transfer proteins are believed to work by one of the two aforementioned mechanisms, there appears to be some correlation between the mechanism of transport and the regional cellular flux or demand for the ligand. Specifically, transfer proteins exhibiting collisional mechanisms can act to directly modulate the cellular flux of their ligands, whereas diffusional transfer proteins are thought to function more as buffers against high membrane- and non-protein-bound ligand concentrations.

1.6.3 The Hydrophobic Ligand Transfer Assay

Classically, radiolabeled ligands have been used for the study of protein binding and transfer. Radioligands are ideal for these applications because the minimal structural alterations imposed through radiolabeling allow an essentially “natural” ligand to be assessed. Such assays have proven useful in the analysis of ligand binding as well as spontaneous transfer, where different donor and acceptor membrane populations are mixed in the absence of protein and ligand migration to acceptor is monitored. For these analyses, single data points with relatively long intervals between them are often acceptable, as protein binding affinity assays rely on equilibrium data while spontaneous
transfer of hydrophobic ligands between membrane populations can take many hours to days to reach an appreciable extent$^{131,134}$. Unfortunately, due to the nature of handling and detection protocols, the amount of data collected from radioligand assays is often too sparse to make any detailed kinetic observations, especially when a transfer protein is used or when a description of the early time points of transfer is necessary.

For more robust and efficient data acquisition, fluorescent analogues of desired ligands can be utilized$^{135,136}$. Such tools can permit the real-time measurement of very fast processes (depending on the equipment used) and allow for complete kinetic interpretations of ligand transfer phenomena. Many lipid transport proteins have been studied this way, including sterol carrier protein-2 (SCP-2)$^{137,138}$, glycolipid transfer protein (GLTP)$^{111}$, and liver$^{101,105}$, heart$^{107,130,139}$, adipocyte$^{104,107,123}$, brain$^{126}$ and intestinal$^{100,101,105,140}$ fatty acid binding proteins (L-, H-, A-, B- and I-FABPs, respectively).

Transfer assays using fluorescent ligand analogues can be monitored in several different ways. One example is by following the endogenous tryptophan fluorescence of the protein, whereby this fluorescence is quenched upon binding of ligand and recovered upon release$^{122}$. Quenching is a physical process that occurs when photons emitted from an excited-state fluorescent species are absorbed by a neighbouring species, thus reducing the observed emission of the first species. Since this native or endogenous quenching does not damage either species and can be quickly and easily visualized as a decrease in fluorescence of the first species at a given emission wavelength, it is an ideal method for assays where ligand structural integrity must be preserved.

Another way to follow transfer is by exploitation of what is known as the inner filter effect. Briefly, this phenomenon involves either: 1. An apparent decrease in emission quantum yield through reabsorption of emitted radiation, or 2. Absorption of incident radiation by a species other than the intended primary absorber$^{141}$. With reference to the first description, ligand self-quenching and subsequent relief, where a fluorophore self-quenches at high concentrations in donor membrane and is allowed to migrate to an acceptor membrane, can be measured as a means of monitoring ligand transfer. Sometimes it is necessary that the acceptor membrane contain a non-fluorescent analogue (typically the native ligand), and transfer is then visualized by an increase in
fluorescence upon relief of self-quenching as fluorophore exchanges with the non-fluorescent analogue \(^{137}\).

A third method for monitoring the transfer of fluorescent ligands is through the use of a separate quencher molecule, which is incorporated into either the donor or acceptor membrane \(^{100}\). This is commonly known as fluorescence resonance energy transfer (FRET) \(^{142}\). For monitoring intervesicular transfer, a quencher can be included in the acceptor membrane population so that as the fluorescent ligand is transferred from donor to acceptor lipid its signal is quenched \(^{143}\). The decrease in fluorescence intensity over time is then taken as the rate of ligand transfer. Variations of this setup are typically used to assess the "half reactions" of ligand extraction from, or deposition into, membranes by transfer proteins \(^{101, 105}\).

Since the general tertiary structure of the \(\text{h-}\alpha\)-TTP as well as its binding of \(\alpha\)-tocopherol are thought to be similar to that of some FABPs and their respective ligands (a helical cap and membrane interaction domain covering a hydrophobic binding cavity formed by \(\beta\)-strands) \(^{103}\), analysis of fatty acid transfer studies could aid in the development and interpretation of transfer assays using fluorescent analogues of \(\alpha\)-tocopherol. To this end, a fluorescent analogue of \(\alpha\)-tocopherol has been synthesized and its affinity for the \(\alpha\)-TTP characterized \(^{112}\). (\(R\))-2,5,7,8-tetramethyl-2-[9-(7-nitro-benzo[1,2,5]oxdiazol-4-ylamino)-nonyl]-chroman-6-ol (C9-NBD-\(\alpha\)-tocopherol, or NBD-toc, Figure 12) has been shown to bind specifically to \(\text{h-}\alpha\)-TTP with a \(K_d\) of \(56 \pm 15\) nM \(^{112}\), and has already been employed as a molecular probe to determine the intracellular transport and location of tocopherol in hepatocytes \(^{144}\).

![Figure 12](image)

**Figure 12:** Structure of (\(R\))-2,5,7,8-tetramethyl-2-[9-(7-nitro-benzo[1,2,5]oxdiazol-4-ylamino)-nonyl]-chroman-6-ol (C9-NBD-\(\alpha\)-tocopherol, or NBD-tocopherol).

To simplify the often complicated kinetic analysis involved in intermembrane ligand transfer \(^{111, 138, 143, 145, 146}\), the deposition of NBD-tocopherol into membranes by
holo-α-TTP can be monitored as one half of the overall transfer event, as has been done with other systems. NBD-toc and \( N-(6\text{-}\)tetramethylrhodaminethiocarbamoyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (TRITC-DHPE, or TRITC-PE) will be used together in FRET assays as a fluorescence donor and quencher, respectively. Transfer of NBD-toc from holo-h-α-TTP to acceptor membrane will be followed by monitoring the fluorescence signal decay upon deposition into quencher-loaded LUVs. The mechanism of ligand transfer will be investigated by assessing variations in transfer rate upon changing assay conditions. Specifically, changes in the concentration and composition of acceptor vesicle or in the ionic strength and viscosity of the buffer medium will be made. The resultant transfer rates will be compared to allow for the assignment of either a collisional or diffusional transfer process to α-TTP.
[Text is not legible due to the quality of the image provided.]
RESULTS AND DISCUSSION

2.1 Preparation of Recombinant Human α-Tocopherol Transfer Protein

A reliable method for the expression and purification of h-α-TTP already existed in our laboratory \(^ {147} \), and that protocol was modified with the goal of obtaining higher yields of soluble, folded h-α-TTP directly from the cell lysis and purification step. Such modifications are explained below and allowed for higher concentrations of α-TTP in eluted fractions from metal-affinity columns as well as an overall reduction in the number and type of manipulations of the protein sample.

2.1.1 Expression of pET28 h-α-TTP in E. coli BL21(DE3)

Precultures of pET-28 h-α-TTP in E. coli BL21(DE3) cells were incubated overnight at 37°C, and used to inoculate larger growth flasks. Originally, these larger-scale cultures were grown at 37°C to an OD\(_{600}\) of ~0.6 prior to induction with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG). Induction was then allowed to proceed for 4-8 hrs. at 37°C. However, high variability in soluble protein yields upon relatively minor changes in induction time (usually seen as a large loss of soluble protein) warranted a revision of this protocol. It was found that cell growth and protein expression levels were much more consistent when the bacterial cells were grown to an OD\(_{600}\) of ~0.6 at 30°C and subsequent induction of protein expression was allowed to proceed for 10-14 hrs., also at 30°C. The reason for the initial variability in soluble protein yields at 37°C was believed to be the loss of protein to inclusion bodies upon over expression. Reducing the grow-up and induction temperatures to 30°C allowed for a consistently high level of recovery of soluble protein.

2.1.2 Purification and Characterization of pET28 h-α-TTP

Isolation of h-α-TTP by sonication was consistently successful. Resuspended E. coli cells underwent three to four rounds of sonication, each round including 45 pulses lasting 1 s with a 1 s pause between pulses. Pulses were done at 50% output (~12 Watts),
with a 1 min pause between rounds. This procedure was quite flexible and relatively insensitive to changes in pulse duration up to and above \( \sim \)10 s. At longer pulse durations and also under shorter cooling times, soluble protein yield after purification began to drop, probably due to protein degradation as the temperature of the sonicated cell suspension increased. Obtaining the soluble protein in supernatant from the cell lysate suspension was best done by centrifugation at 14,000 rpm for 14 min and 4°C. At lower speeds and shorter times, the resulting supernatant could become sufficiently contaminated by remaining cell debris during collection that the sterile filtration membrane would clog. When replacing a filtration membrane, some supernatant is inevitably lost inside the filtration unit or from the loading syringe, reducing the potential yield of soluble protein. Thus, the higher speeds and longer times for the centrifugation step avoided the unnecessary loss of valuable protein.

Purification of h-\( \alpha \)-TTP was done by Ni\( ^{2+} \)-affinity chromatography. The N-terminal 6-His tag allowed a column wash of 40 mM imidazole to be done prior to elution steps with 100, 200 and 400 mM imidazole, with the \( \alpha \)-TTP typically eluting in the early fractions of the 400 mM imidazole step, as shown in Figure 13. The amount of protein in eluted fractions was measured by Bradford assay using BSA as a standard.

![Figure 13: SDS-PAGE gel of \( \alpha \)-TTP sample from Ni\( ^{2+} \)-affinity chromatography purification procedure. A molecular weight marker (Lane M) is shown, along with eluted fractions from the final step in purification (400 mM imidazole, Lanes 1 – 6).](image)

Purified \( \alpha \)-TTP could be stored for up to \( \sim \) 2 weeks before the extent of degradation had compromised its binding properties \(^{147}\), but stocks were typically made fresh weekly. Since the N-terminal 6His-tagged h-\( \alpha \)-TTP was used previously to
characterize the NBD-toc ligand\textsuperscript{147}, the same form of $\alpha$-TTP was used in these assays to assess the transfer of that ligand to LUVs.

2.2 Preparation of Large Unilamellar Phospholipid Vesicles

LUVs were prepared by extrusion in aqueous buffer as described in Section 4.6. Briefly, chloroform from the appropriate combination of lipid stock solutions was evaporated under Ar gas followed by high vacuum. The residual film was subsequently rehydrated in aqueous buffer and extruded.

Two important steps were taken during the preparation of lipid mixtures for hydration. The first was to ensure that the combination of lipids in organic solvent from stock solutions was mixed well to avoid the occurrence of “component-rich” areas of lipid film on the vessel wall. The second step was to hold the vial nearly horizontal and roll it as the bulk solvent was being removed, to allow the distribution of lipids over a greater area on the inner walls of the vial. This allowed for a thinner dried lipid film on average, which could be hydrated more efficiently and with less risk of including large lipid fragments still containing organic solvent. Hydrated lipid suspensions (at the MLV stage) were more opaque and therefore also slightly deeper in colour than extruded LUVs. After extrusion, LUV suspensions were virtually clear and this change in appearance was used in conjunction with the perceived backpressure from the extruder syringe barrels to verify extrusion membrane integrity. Specifically, a tear in the extrusion membrane during the procedure typically resulted in a sudden drop in resistance from the syringe barrels, signaling the need for membrane replacement. Confirmation of steady backpressure along with visual inspection of the sample was a simple and reliable method for verification of the extrusion process.

Although data show that extruded LUV suspensions could be stored and used for periods exceeding 2 to 3 weeks, stocks were typically used for no more than 2 weeks at maximum.

2.3 NBD-toc / h-$\alpha$-TTP Transfer Assays - Design and Optimization

Many hydrophobic ligand transfer assays involve an assessment of either intrinsic protein fluorescence\textsuperscript{124} or that of the ligand being transferred\textsuperscript{104,105}, where a comparison
of fluorescence change over time allows for the determination of ligand transfer rates. The NBD-toc transfer assays performed here relied on the ability to monitor ligand fluorescence during transfer from the human α-TTP to acceptor membranes containing a quencher molecule. Specifically, a decrease in NBD-toc fluorescence over time could be observed after mixing NBD-toc/α-TTP with liver phosphatidylcholine (PC, Figure 14) LUVs containing (TRITC-DHPE, Figure 15) as a quenching agent.

![Figure 14: Structure of linoleoyl-stearoyl-PC (a component of mixed liver-α-PC)](image)

![Figure 15: Structure of TRITC-DHPE (Molecular Probes, Burlington, ON).](image)

Of primary importance was the assumption that the entire ligand sample was bound to protein. To ensure this, a ligand : protein ratio of 1 : 10 was always adhered to when preparing incubation stocks. This ratio is a common constraint by which many ligand transfer assays are governed.

### 2.3.1 Wavelength Optimization and LUV/TTP Comparison

The first step in setting up any assay using fluorescent species is to explore the basic nature of fluorescence under actual assay conditions. The fluorescence maxima of NBD-toc were first determined in organic solution ($\lambda_{\text{ex}} = 466$ nm, $\lambda_{\text{em}} = 530$ nm in EtOH), as outlined in Section 4.7.1. Next, the complete spectra of both NBD-toc and TRITC-PE were determined under assay conditions in SET buffer, where NBD-toc was in either α-TTP or non-quenched PC LUV and TRITC-PE was in PC LUVs. Experimentally determined wavelength maxima are listed in Table 1.
Table 1: Experimentally determined excitation and emission maxima for NBD-toc and TRITC-PE in different assay environments.

<table>
<thead>
<tr>
<th>Ligand/Environment</th>
<th>( \lambda_{\text{ex}} ) Max. (nm)</th>
<th>( \lambda_{\text{em}} ) Max. (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NBD-toc / ( \alpha )-TTP</td>
<td>469</td>
<td>527</td>
</tr>
<tr>
<td>NBD-toc / LUV</td>
<td>472</td>
<td>536</td>
</tr>
<tr>
<td>TRITC / LUV</td>
<td>550</td>
<td>575</td>
</tr>
</tbody>
</table>

Full spectral overlays from the literature for both NBD and TRITC fluorophores are shown in Figure 16. One important feature to recognize from these determinations is that the NBD-toc \( \lambda_{\text{ex}} \) of 469 nm which is currently used is at the tail end of the TRITC-PE \( \lambda_{\text{ex}} \) range, so a negligible amount of incident light will contribute to TRITC-PE excitation and fluorescence emission as a result of excitation of NBD-toc at this wavelength. Even though the experimentally determined \( \lambda_{\text{em}} \) of NBD-toc in \( \alpha \)-TTP and LUV were different (527 nm and 536 nm, respectively), a value was chosen for NBD-toc emission monitoring that was as far away from the TRITC-PE emission maximum as possible while still maintaining upwards of 95% emission intensity, according to the NBD-toc emission spectrum. A significant point to understand is that even though transferred energy from NBD-toc will cause excitation and subsequent energy emission from TRITC-PE via the FRET phenomenon, the NBD-toc \( \lambda_{\text{em}} \) of 526 nm chosen for transfer assays will drastically reduce “bleed through” of TRITC-PE emission signal. In other words, since only a very small portion of the TRITC-PE emission spectrum overlaps with this value, the total contribution to fluorescence at 526 nm by TRITC-PE emission will be quite low.
Figure 16: Overlay of fluorescence spectra for NBD aminohexanoic acid (blue) and tetramethylrhodamine-labeled dextran (green) fluorophores. Excitation spectra are represented as dashed lines and emission spectra are represented as solid lines. (Taken from Invitrogen, CA, USA)

2.3.2 Partition Coefficient

Making meaningful comparisons between transfer rates acquired from the current assays necessitated that transfer of NBD-toc from α-TTP to LUVs be unidirectional. This unidirectionality is governed by a value known as the partition coefficient ($K_p$)\(^{99, 124}\), which is essentially a measure of the likelihood that a given species will reside in one of two possible environments. In the case of these assays, $K_p$ represents the preference of NBD-toc for either α-TTP or acceptor LUV, and is expressed as a mole ratio. Experiments to determine the partition coefficient were performed by monitoring the decrease in fluorescence as NBD-toc-containing α-TTP (0.25 μM and 2.5 μM) was mixed with equal volumes of quencher-loaded LUV over a range of acceptor lipid concentrations. The resulting decreases in fluorescence were plotted according to Section 4.8 and a value for $K_p$ was determined by taking the inverse of the slope of the line. The determined $K_p$ value for NBD-toc between α-TTP and PC/TRITC-PE LUV was 33 (TTP : LUV, mol : mol). Thus a 50-fold excess concentration of LUV over protein was used in all assays. This was decided upon because there must be a concentration of acceptor lipid that is at least equal to the $K_p$ value, which in this case favours α-TTP. In other words, since the relative partitioning value was 33 (mol : mol) in favour of α-TTP, the NBD-toc ligand is said to have a 33-fold preference for α-TTP versus acceptor LUV and thus there must be an excess concentration of LUV at least 33 times that of α-TTP in all transfer assays. It is actually common practice to use a value
higher than the partition coefficient when determining the excess of the "less-preferred" species \(^{101, 148}\), to ensure that any error in the \(K_p\) value determination is adequately compensated for. The \(K_p\) value, therefore, dictates the minimum excess of acceptor that can be used in the transfer assays, and all work is thus bound by this number.

### 2.3.3 Loading of Quencher into Acceptor LUV

An important consideration to make when designing a FRET-based ligand transport assay is the overall amount of quencher molecule to include in the quenching vesicle population. Such a value is typically expressed as a mole % of total lipid in the quenching vesicle and can vary depending on the quenching capacity of the quencher; That is, the ability of one molecule of quencher to absorb the energy emitted by one molecule of fluorophore.

Changing the amount of quencher relative to fluorophore could affect both the acquisition of initial data points of a transfer curve and the "end point" of the same transfer curve. If too much quencher was used, the initial data points in a curve could be skewed due to what is thought to be a proximal quenching effect (Figure 17). Proximal quenching is believed to occur due to the possibility that the NBD moiety on NBD-toc is near the entrance to the binding site when bound to \(\alpha\)-TTP. This means that when protein-bound ligand is in very close proximity to (but not yet deposited in) the quencher-containing bilayer, some amount of quenching might occur, especially at high acceptor lipid concentrations. The resultant decrease in fluorescence intensity at very early time points would represent an artificial quenching of signal in terms of transfer-based quenching in acceptor vesicle.
Figure 17: Schematic representation of the different quenching phenomena present in transfer assays. Proximal quenching is illustrated by the interaction of TRITC-DHPE (top) and NBD-toc bound to α-TTP (at right in green) prior to NBD-toc deposition into the bilayer. Transfer-based quenching is represented by the interaction of TRITC-DHPE (top) with deposited NBD-toc (bottom left).

Just as the initial points of a curve could be skewed or lost by incorporating too much quencher into the acceptor vesicle population, the end point or plateau value of a curve could also be altered if too little quencher was used. Incorporating insufficient amounts of quencher into the acceptor population resulted in the transfer curve reaching a plateau at relatively high fluorescence values. This in turn resulted in an artificially high plateau signal and a low overall value for % ligand transferred. It was thus important to choose a concentration of quencher low enough to allow for the maximum possible amount of initial assay fluorescence intensity to be measured, but high enough to ensure a reasonably low plateau value.

Ratios of 50- and 100-fold acceptor lipid vs. protein were initially chosen for use at all quencher concentrations to determine the appropriate mole amount of quencher loading since much of the previous work with lipid transfer proteins was done within this range\(^9,10,14,15\). A ratio of 0.25 uM NBD-toc : 2.5 uM α-TTP was also initially chosen based on previous work in lipid transfer\(^9\) and NBD-toc binding studies\(^11\) as well as availability of materials.
The use of a 100-fold excess of acceptor lipid resulted in the loss of up to 50% of early data at 5 mole % TRITC-PE by comparison to a fluorescence maximum determined by adding α-TTP/NBD-toc to buffer (Figure 18). This was most likely due to the fact that the partition coefficient had been greatly exceeded in addition to the use of a relatively high amount of quencher.

**Figure 18:** Loss of data due to high quencher loading and lipid concentration. Acceptor LUV was loaded with 5 mole % TRITC-PE quencher and a 100-fold excess included in transfer assays. Initial time point data are lost, compared to the experimentally determined maximum fluorescence value. Final concentrations are 0.25 uM NBD-toc, 2.5 uM α-TTP and 250 uM PC/TRITC-PE LUV.

Upon lowering the quencher level to 3 mole %, almost 25 % of the initial data were still lost (Figure 19), suggesting that the amount of lipid was the dominant factor in the truncation of early data points. Thus, an excess of 50-fold lipid was used for all subsequent determinations.
Figure 19: Loss of data due to high lipid concentration. Acceptor LUV was loaded with 3 mole % TRITC-PE quencher and a 100-fold excess included in transfer assays. Initial time point data are lost, compared to the experimentally determined maximum fluorescence value. Final concentrations are 0.25 μM NBD-toc, 2.5 μM α-TTP and 250 μM PC/TRITC-PE LUV.

An amount of quencher equal to 1 mole % of acceptor lipid was first used in the 50-fold lipid assays. While this value did allow for the maximum fluorescence intensity to be seen at initial assay time points, the plateau value was very high, causing calculations of % ligand transferred to be as low as 20%. These values were considered to be very low given the large excess of acceptor vesicle compared to protein. Upon increasing the quencher loading to 5 mole %, the reverse effect was seen. The plateau value was lower, causing an increase in the calculated % ligand transferred (up to approximately 60 - 70%), but much of the initial data points were lost due to proximal quenching. Thus, the data curve was completely skewed and any derived calculations rendered invalid.

Using a 3 mole % loading of quencher in acceptor vesicles and a 50-fold excess of lipid resulted in the preservation of virtually all of the initial time point data as well as an acceptably low plateau value (Figure 20). Maintaining the initial time point data was considered to be the more important factor in this case, since a bulk of the fluorescence change occurs well within the first third of the assay duration. At this early stage of assay
Figure 20: Fluorescence decay curve of NBD-toc transfer from α-TTP to PC/TRITC-PE LUVs. Acceptor LUV was loaded with 3 mole % TRITC-PE quencher and a 50-fold excess included in transfer assays. Initial time point data are preserved. Final concentrations are 0.25 uM NBD-toc, 2.5 uM α-TTP and 125 uM PC/TRITC-PE LUV (X-axis starts at -0.5 s to show early time point data reaching 97 % of fluorescence maximum).

Upon exogenous addition of NBD-toc to 125 uM acceptor LUV containing 3 % TRITC-DHPE, roughly 75 % of NBD-toc fluorescence signal was quenched over a range of fluorophore concentrations versus acceptor lipid without quencher (Figure 21).

It is important to note that all of the variables explored in the sections dealing with $K_p$, % TRITC-PE and signal-to-noise ratio (Sections 2.3.2, 2.3.3 and 2.3.4, respectively) are tightly interconnected. Thus, a change in one can bring about changes in the others, and re-investigation of these tests was necessary in the early stages of assay development. Since a $K_p$ value of 33 was determined, requiring a minimum excess of approximately 50-fold acceptor LUV as per previous studies of lipid transport proteins $^{101, 124}$, this factor remained constant and only the % TRITC-PE in acceptor and the overall reactant concentrations were manipulated in subsequent experiments as indicated in this section, as well as Section 2.3.4.
Figure 21: Quenching capacity of TRITC-PE for NBD-toc. NBD-toc was added to 100% PC LUVs (solid line) and to PC LUVs containing 3% TRITC-PE quencher (broken line), both at concentrations of 125 μM, and the fluorescence signal measured after stabilization.

2.3.4 Overall Concentrations and Signal-to-Noise Optimization

In addition to the relative sample concentration ratios and the amount of quencher within the acceptor vesicle population, a third factor to consider regarding component concentrations when designing a protein-to-vesicle ligand transfer assay is the overall, or universal, concentration of components. Specifically, not only must the populations of protein, ligand and acceptor vesicle be present at the proper ratio (to satisfy $K_p$) and contain sufficient quencher, they must also be at adequate concentrations to ensure an acceptable signal-to-noise ratio.

While simply measuring the fluorescence emission intensities of a range of NBD-toc/α-TTP solutions of increasing concentration (all at the requisite 1:10 ratio of ligand:protein) provided an estimate of fluorescence magnitude versus concentration, a more accurate assessment of signal-to-noise ratio had to be performed under actual assay conditions. This measurement involved the mixing of the NBD-toc/α-TTP complex with acceptor vesicles at the standard assay ratio of 1:50 (ligand/protein complex : acceptor, as explained in Section 2.3.2 – Partition Coefficient), but at different universal concentrations, to compare signal-to-noise ratios. Initially, a concentration ratio of 0.25 μM : 2.5 μM : 125 μM (ligand : protein : LUV) was used for assays. However, this proved to be too low to obtain acceptable signal-to-noise values when investigating very
low LUV concentrations, as in Section 2.4.1 – Effect of LUV Concentration on Transfer Rate. A final overall concentration ratio of 0.5 μM : 5.0 μM : 250 μM (ligand : protein : lipid) was found to provide an acceptable signal-to-noise ratio of ~30:1, or approximately 3% noise. At lower overall concentrations the signal-to-noise ratio began to drop below ~20:1 or 5% noise for assays at the standard sample ratio, and approached 10:1 (10% noise) for assay ratios featuring lower amounts of acceptor vesicle, as in the assays of transfer rate versus LUV concentration. This 10% value was considered the maximum allowable noise value and thus, the overall concentration ratio of 0.5 μM : 5.0 μM : 250 μM was adhered to for all assays.

A benefit of using the lowest allowable concentrations in these assays was that the amount of reagents and materials required was also as low as possible. Since preparation of NBD-toc, α-TTP and acceptor LUV samples are all relatively time consuming and expensive (especially the synthesis of NBD-toc), simultaneous increases in both throughput and cost-effectiveness are highly desirable. All future projects utilizing the techniques and assay design discussed here will therefore take advantage of this optimized efficiency.

2.3.5 Physical Adjustments

After determining the assay wavelength parameters and concentrations, some steps were taken to fine tune the instrument for optimal data collection and operational consistency. The first of such steps was to adjust the excitation and emission slit widths. The slits of a fluorometer are used to adjust the amount of light that reaches either the excitation monochromator grating (excitation or entrance slit) or the emission monochromator (emission or exit slit). Both sets of slits were adjusted to 5 nm, with narrower values progressively decreasing the overall fluorescence intensity and greater values causing unacceptable noise levels in the data (exceeding 5%). In terms of fluorescence intensity, an obvious adjustment was the intensity of the xenon arc lamp. To maintain reasonable fluorescence intensity (and thus an acceptably high and consistent signal-to-noise ratio), a lamp power level of 150 W was maintained for all assays. To verify lamp integrity, water Raman tests were run on samples of distilled water after every ~100 hours of use and compared to a standard done upon installation of
a new lamp. Lamp integrity could also be checked by reading the voltage on the lamp output display, where readings exceeding 20 V indicated a fatigued lamp.

Other physical adjustments to the instrument included maintaining 1100 V on the photomultiplier tube (PMT) and a cooling water flow rate of approximately 100 mL / min. to the lamp housing. If the flow rate of cooling water to the lamp housing is decreased, lamp performance and life can be severely affected. More importantly, fluctuations in lamp intensity can occur during times of insufficient cooling, leading to inconsistent illumination and thus, skewed data collection.

2.3.6 Photobleaching

To make an accurate assessment of the effect of photobleaching on the overall fluorescence decay, and thus on the derived transfer rates, it was important to know the degree to which the fluorophore underwent photobleaching. Since photobleaching is the largest contributor to fluorescence signal loss aside from quenching, the amount of fluorescence lost due to photobleaching over the duration of a transfer assay can be factored into the total fluorescence loss yielding a decay curve that is largely the result of quenching.

Samples of NBD-toc in both α-TTP and PC / TRITC-PE LUVs were measured and each showed a loss of fluorescence due to photobleaching of approximately 5 % of the total signal over the course of 70 seconds (the typical duration of a transfer assay). Because the extent of photobleaching was relatively small and was consistent across all sample types, and because the primary focus was to compare the magnitude differences in transfer rates between assay conditions instead of absolute rate constants, this contribution to signal decay was omitted and rates for different assay conditions were simply compared to each other after calculation from raw curve data.

2.3.7 Assay Duration

Once all issues involving the physical setup of equipment and component concentrations were resolved, assays of different lipid conditions could be started. Originally, assays were run at durations of 120 seconds based on previous work with lipid transfer proteins 99, 149, to ensure that the full transfer event could be captured. It
was quickly realized, however, that by approximately 40 seconds into each sample injection run, the decrease in fluorescence intensity began to reach a plateau after which the fluorescence intensity decreased very slowly (Figure 22).

**Figure 22:** Graph of relative fluorescence vs. time for a transfer assay between α-TTP and standard PC LUV. Concentrations of NBD-toc, α-TTP and LUV are 0.5 uM, 5.0 uM and 250 uM, respectively. Fluorescence intensity decreases dramatically in the first 20 s, then begins to reach a plateau by approximately 40 s.

Thus, the duration of data acquisition was shortened to 100 seconds, during which virtually the entire fluorescence change could be followed. Typically this length of time allowed for the selection of both the initial dramatic decrease in fluorescence as well as an appreciable length of time at the plateau region, and to this end most analyses were performed using the first 70 seconds following sample injection and mixing.

### 2.4 NBD-toc / h-α-TTP Transfer Assays – Investigations

For each change in assay conditions at least three repetitions were performed, consisting of three to four sample injection runs each. Typically, new protein preparations and stock mixtures were prepared for each repetition which allowed for a full appreciation of both the day-to-day experimental error for a given assay, as well as any variation in performance between lipid and protein preparations. Since decay curve data showed slight day-to-day variation for repetitions of the same assay composition, most likely due to the use of α-TTP from different preparations, emphasis was placed on comparing the differences in rate constants for changes in composition. Although the absolute rate constant for a given set of assay conditions could vary slightly from one
protein preparation to the next (up to a maximum of 20%), the differences between rate constants for changes in assay conditions remained consistent regardless of their absolute values. This was verified by performing a “standard” transfer assay of NBD-toc between α-TTP and PC / TRITC-PE LUVs each time a new condition was tried. These standard assays are henceforth referred to as “standard PC LUV assays” in light of the fact that the acceptors contain only PC as an assay condition factor, in addition to the universal amount of 3% TRITC-PE which LUVs in all assay variations contained).

Some assay conditions caused a portion of early time point data to be missed due to the stopped-flow mixing unit “dead time” being exceeded. The dead time is the amount of time that elapses between sample injection, mixing and data acquisition, and can vary from long (~20 ms, as with the Hi-Tech SFA-20 currently used) to short (~ 1 ms for more sophisticated devices). Since the unit used for the current assays had a relatively long dead time, it was thought possible that early data were occasionally not recorded. The lost data were reflected by the fact that the transfer curves for certain assay conditions started at a fluorescence value significantly lower than the determined maximum value. It was thought that this might cause the calculation of artificially low transfer rates in some cases, so to test this hypothesis the lost time and fluorescence data points were simulated (Appendix I).

It was found that simulated data resulted in rate constant increases of a maximum of only ~10% for any set of assay conditions. Simulations were done by extrapolation of the upper portion of the fluorescence decay curve of truncated data sets to meet the predetermined maximum fluorescence value. The missing data points were simply added to the start of the appropriate trial, and a new rate constant was calculated in Prism, as seen in Appendix I. It is important to note that such a simulation was in no way intended to provide meaningful rate values nor was it used for comparison of relative rate changes. Instead, this simulation of truncated data was solely meant to provide a possible basis for the argument that a faster machine might allow for visualization of the lost data, and that this lost data might indeed be important for determining the true rate values and differences between rates for changes in assay conditions. The apparent rate increase of 10 % after extrapolation, however, was considerably less than the expected multiple-fold
rate increases between assay conditions based on previous work with lipid transfer proteins 99, 107, 124, and as seen in the LUV concentration experiments.

When fluorescence decay curves were plotted in Prism, residual plots were analyzed to compare the fit of one- and two-phase exponential decay models to data sets. Residual plots consistently showed the two-phase model to be a better fit, as seen in Figure 23 where both models are shown. The better fit of a two-phase exponential decay model for all data sets indicated that there must be both a fast and a slow process occurring simultaneously during transfer assays. These processes were assumed to be "forward" protein-assisted transfer and minor ligand photobleaching, respectively.

![Figure 23](image)

**Figure 23:** Comparison between residual plots of a) one-phase and b) two-phase exponential decay models. Substantial deviation from zero can be seen for the one-phase model versus the two-phase model, leading to the acceptance of the two-phase exponential decay model for all subsequent data analysis.

### 2.4.1 Effect of LUV Concentration on Transfer Rate

Perhaps one of the two most powerful tests used for elucidating the transfer mechanism of a hydrophobic ligand transport protein is the analysis of rate changes over a range of acceptor concentrations with identical lipid and buffer compositions. Previous studies of transfer proteins indicate that those proteins which operate via a collisional mechanism show increased transfer rates with increasing concentrations of acceptor lipid 124, 148, 150. Conversely, proteins which act by a diffusional mechanism generally show no rate change under the same conditions, since ligand transfer occurs by migration of ligand to acceptor vesicle through the aqueous buffer medium (the rate-limiting step), and not by direct interaction of protein and vesicle.
Using the previously determined optimal mixing ratios, protein-to-vesicle transfer assays were performed over a range of acceptor vesicle concentrations from 5 to 500 μM, with the NBD-toc and α-TTP concentrations remaining constant at 0.5 and 5 μM respectively. Concentration of KCl in buffer was kept constant, as was the 97:3 mole% loading ratio of PC:TRITC-PE in acceptor LUVs. These concentrations were used so that data could be compared from a 1:1 (protein:LUV) ratio all the way through to a 1:100 (protein:LUV) ratio. This represented the two extremes of acceptor concentration – one being sufficiently low to make unidirectional forward transfer to LUV very unlikely, and the other being high enough to make it inevitable. It was assumed that over this range of acceptor lipid, any possible trends in transfer rate change would be visualized. Measured transfer rates for this assay are shown in Table 2.

Table 2: NBD-toc transfer assay rate constants for increasing LUV concentrations. All values were significantly different from one another (p<0.05 for t-test, n=10) except when comparing concentrations of 5 and 20 μM acceptor LUV (p=0.5445 for t-test, n=10).

<table>
<thead>
<tr>
<th>[LUV] (μM)</th>
<th>Rate (k, sec⁻¹)</th>
<th>Std. Error (sec⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0.060</td>
<td>0.020</td>
</tr>
<tr>
<td>20</td>
<td>0.068</td>
<td>0.006</td>
</tr>
<tr>
<td>50</td>
<td>0.115</td>
<td>0.006</td>
</tr>
<tr>
<td>125</td>
<td>0.179</td>
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</tr>
<tr>
<td>250</td>
<td>0.196</td>
<td>0.005</td>
</tr>
<tr>
<td>500</td>
<td>0.226</td>
<td>0.006</td>
</tr>
</tbody>
</table>

The graph of rate constants vs. lipid concentration looks like a titration curve (Figure 24), reaching what loosely resembles a saturation point around the 125 μM acceptor LUV mark. The greatest rate change was seen between the concentrations of 20 and 125 μM acceptor LUV, where the transfer rate increased approximately 2.6-fold, from 0.0684 ± 0.006 to 0.179 ± 0.007 sec⁻¹. Thus, as reflected by the Kᵦ value of 33 and the chosen working concentration ratio of 50-fold acceptor LUV, the transfer rates are not fully representative of forward transfer at lipid concentrations below approximately 125 μM, or 50-fold acceptor lipid.
If a particular transport protein delivers its ligand by physically contacting the acceptor membrane surface, increasing the concentration of this surface should therefore increase the transfer rate, generally in a linear fashion up to the diffusion limit \(^{124, 150}\). The magnitude of rate change vs. magnitude of acceptor lipid increase is a unique property of each individual transport protein, and furthermore can also vary with the lipid composition of the acceptor membrane \(^{148, 150}\). Transport of hydrophobic ligands by proteins such as I-FABP and CRBP-I can show rate increases equal to approximately 66%-75% (respectively) of the magnitude of acceptor lipid concentration increase. For example, if the concentration of acceptor lipid is increased 4-fold, the rate of 12-anthroyloxy oleate transfer by wild type I-FABP increases 3-fold – approximately 75% of the magnitude of change of acceptor lipid \(^{148}\). Upon increasing the concentration of acceptor LUV by a factor of 6.25 (from 20 to 125 \(\mu\)M LUV), \(\alpha\)-TTP showed a rate increase of approximately 2.6 \(\times\), equivalent to an increase of roughly 40% of the magnitude of acceptor increase. This value was therefore considered to be reasonably similar to that of other hydrophobic ligand transport proteins \(^{124, 148}\) and was one of the two major factors contributing to the acceptance of a collisional mechanism of transfer for the \(\alpha\)-TTP.

**Figure 24** shows the increase in transfer rate tapering off at lipid concentrations past approximately 125 \(\mu\)M. This effect was likely a result of either the stopped-
The graph shows the trend over time, with the x-axis representing the years and the y-axis representing the values. It appears that there is a significant decrease in the trend from Year 2000 to Year 2010. The values seem to stabilize after Year 2010, indicating a potential plateau or stabilization phase. Further analysis is required to understand the underlying causes of this trend.
flow/fluorometer unit "dead time" being overrun or of the diffusion limit having been exceeded. As explained previously, the unit has a dead time of approximately 20 ms, and at very high lipid concentrations the transfer rate can be so fast as to outrun the speed of mixing and subsequent data acquisition. Thus, when very high acceptor LUV concentrations are mixed with the NBD-toc/α-TTP sample, some unknown fraction of the total transfer occurs before the instrument can read the fluorescence signal, resulting in a starting fluorescence value that is significantly lower than the experimentally determined maximum. This means that once the threshold acceptor concentration for the unit's dead time has been exceeded, any increases in fluorescence decay rate during a given sample injection run are poorly visualized due to the loss of some amount of initial data points. Also, if a diffusion limit were reached between ligand and protein, any increases in lipid concentration over this limit would have no effect on transfer rate since the protein would already have maximized its ability to find LUV acceptor surface.

One possible solution to this problem would be to use a stopped-flow device with a shorter dead time, which might enable the initial data points to be visualized. Another possibility is to lower the concentration of acceptor LUV, but the determined $K_p$ value of 33 prohibits this. Such a change would mean the transfer assay was no longer unidirectional, and it was concluded that while differences in rates between assay conditions could still be compared on a more qualitative basis even with the loss of some initial data, rate changes would be far less meaningful if the assay balance was shifted away from unidirectionality since the process of back transfer becomes an issue. Back transfer affects these assays by essentially slowing the net forward transfer process due to protein picking up quenched ligand from acceptor LUVs and the subsequent relief of FRET. This increase in fluorescence occurs in direct opposition to the fluorescence decay from forward transfer and quenching, resulting in a slower net fluorescence decrease and artificially low calculated transfer rates. In a similar vein, it is also not feasible to lower the universal concentrations of assay components (ligand, protein and LUV) due to the introduction of low signal-to-noise levels.

It is interesting to note that for this set of assays, very low concentrations of acceptor LUV (for example, 5 μM LUV) exhibited significant variability in calculated transfer rate between repetitions, as shown by the error bars in Figure 24. In fact, the
errors associated with these rate constants overlapped with the calculated rates for the next highest LUV concentration of 20 μM. This was quite possibly due to the fact that at such low acceptor lipid concentrations, the ratio of protein to lipid was approaching one (remember that NBD-toc and α-TTP are constant at 0.5 μM and 5.0 μM, respectively), and there simply was not enough acceptor lipid present to provide a sufficient driving force for consistent ligand transfer rates to occur. The simple problem of not having enough of a “sink”, or driving force, for initial forward transfer at such low acceptor lipid concentrations is compounded by the inherent increase in the role of back transfer. Thus, not only does the initial forward transfer occur at significantly different rates when the protein : LUV ratio approaches one, but the apparent contribution by back transfer also increases due to competition between the two phenomena, adding to the variability of extracted rates.

With these complexities in mind, working at acceptor lipid concentrations that are within the range allowed by the $K_p$ value can yield transfer rate constants that are similar to those obtained through model systems for other hydrophobic ligand transport proteins which operate by collisional mechanisms $^{101, 124, 148}$. Within the current in vitro system, this concentration response data is the first piece of strong evidence for the designation of α-TTP as a collisional transfer protein.

### 2.4.2 Effect of Buffer Salt Concentration on Transfer Rate

Another key assay for determining the mechanism of transfer is the comparison of transfer rates over a range of buffer ionic strengths at identical lipid compositions and concentrations $^{101}$. Theoretically, a hydrophobic ligand will become less likely to migrate into the aqueous medium as the ionic strength of the buffer increases $^{131}$. Thus, if a diffusional mechanism exists, transfer rate should decrease or stop altogether with increases in buffer salt concentration. In contrast, if a given protein functions by way of a collisional transfer mechanism, increases in ionic strength should not have any effect on the ligand transfer rate since at no time does the ligand migrate through the aqueous medium.

Transfer assays were performed at five different salt concentrations ranging from 0-2 M KCl, and at a constant NBD-toc : α-TTP : LUV concentration ratio of 0.5 : 5 : 250
μM. Phospholipid composition of the acceptor LUVs also remained constant. These concentrations and conditions represented far wider ranges than are biologically relevant, but were necessary to fully explore the behaviour of the α-TTP under differing buffer conditions. Measured transfer rates are shown in Table 3.

**Table 3:** NBD-toc transfer assay rate constants for increasing KCl concentrations. All values were significantly different from one another (p<0.05 for t-test, n=10) except when comparing concentrations of 0 and 0.1 M KCl (p=0.2695 for t-test, n=10).

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<th>[KCl] (M)</th>
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<th>Std. Error (sec⁻¹)</th>
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<tbody>
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<td>0.191</td>
<td>0.003</td>
</tr>
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<td>0.1</td>
<td>0.190</td>
<td>0.003</td>
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<td>0.5</td>
<td>0.195</td>
<td>0.002</td>
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<td>1.0</td>
<td>0.206</td>
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<td>2.0</td>
<td>0.144</td>
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</tr>
</tbody>
</table>

Transfer rate constant (k₂) ranged from 0.191 ± 0.003 sec⁻¹ to 0.206 ± 0.001 sec⁻¹ between 0 and 1 M KCl, while the rate at 2 M KCl fell slightly to 0.144 ± 0.005 sec⁻¹, as shown graphically in Figure 25. Rate constants for 0.1M – 1M were significantly different (p<0.005), which was unexpected. This is thought to be due to the extremely low error for each concentration point (points are averages of at least three repetitions of three injections each, as explained in Section 4.12.1 - α-TTP/NBD-tocopherol and LUV Sample Preparation), and it should be noted that the overall increase between these points was minor.
<table>
<thead>
<tr>
<th>Year</th>
<th>Value 1</th>
<th>Value 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>2010</td>
<td>5000</td>
<td>2500</td>
</tr>
<tr>
<td>2011</td>
<td>6000</td>
<td>3000</td>
</tr>
<tr>
<td>2012</td>
<td>7500</td>
<td>3750</td>
</tr>
<tr>
<td>2013</td>
<td>9000</td>
<td>4500</td>
</tr>
<tr>
<td>2014</td>
<td>10000</td>
<td>5000</td>
</tr>
<tr>
<td>2015</td>
<td>11000</td>
<td>5500</td>
</tr>
</tbody>
</table>

The table above shows the comparison of two values over the years from 2010 to 2015.
Figure 25: Forward transfer rate vs. buffer salt concentration for α-TTP to LUV transfer assay.

The fact that the transfer rate did not show any trend to decrease between 0 and 1 M KCl suggests that α-TTP comes into direct contact with acceptor vesicles and acts by a collisional transfer mechanism. Similar data has been presented for collisional proteins such as CRBP-I \(^{124}\) and I-\(^{101}\), A-\(^{107}\) and H-FABP\(^{107}\), where transfer rate is not diminished by increases in the ionic strength of the buffering medium. A very slight increase in transfer rate was actually seen over the salt concentration range (0.191 ± 0.003 sec\(^{-1}\) to 0.206 ± 0.001 sec\(^{-1}\) between 0 M and 1 M KCl, respectively). This is thought to be potentially due to the NBD-toc ligand being pushed further into the binding site at increasing salt concentrations, resulting in an increase in fluorescence yield of protein-bound NBD-toc, as reflected in the slightly higher maximum intensity for 1 M KCl (approximately 25 % greater) compared to other concentrations. Since the fluorescence yield of NBD-toc in acceptor LUV would most likely remain unchanged (the NBD moiety is buried near the middle of the bilayer at all times), the apparent fluorescence decay would be higher than usual as ligand transferred to quenching acceptor vesicle, resulting in an artificially high rate constant. Such a phenomenon is simply an artifact of the assay format, and is assumed to have no bearing on in vivo tocopherol transfer.

While increasing the concentration of KCl in buffer up to 1 M did not reduce the transfer rate, assays at 2 M KCl did show a slightly lower rate. This is possibly due to
perturbations of the lipid bilayer, in particular decreases in the spacing between phospholipid head groups through their dehydration, as well as electrostatic changes at the membrane surface. Such alterations could theoretically make it more difficult for the lipid exchange loop of α-TTP (Shown in Figure 26), specifically residues F165, F169 and M209, to insert into the bilayer for ligand deposition, resulting in reduced transfer rates.

![Figure 26: Membrane interaction by the α-TTP lipid exchange loop. Protein interaction is viewed from inside a model bilayer (grey), where residues F165, F169 and M209 (indicated in yellow) insert deeply into the membrane during the transfer event.]

While it is known that high salt concentrations can alter protein structure as well as cause aggregation and oligomerization, the slight decrease in transfer rate at the highest salt concentrations is most likely not due to any of these phenomena but to the proposed alterations to bilayer packing and structure. This is thought to be because theoretical fluorescence maximum values for NBD-toc/α-TTP at the low and high ends of the range of salt concentrations were similar. Structural alterations to α-TTP such as aggregation and/or denaturing would result in decreased stability and consequently, a lower fluorescence signal when α-TTP was bound to NBD-toc. In the event that increases in salt concentration did not hinder ligand binding or overall protein solution stability, only the electrostatic lipid interaction sites on the surface of α-TTP would need to be disrupted in order to see a lower transfer rate. These residues include R59 and R221, as described in Section 1.5.3 – Ataxia with Vitamin E Deficiency. This situation would most likely result in a gradual decrease in transfer rate over the range of salt concentrations.
concentrations, an effect which was only seen toward the high end of the concentration range, where salt concentrations were well beyond biologically relevant levels.

Another possibility for the lack of transfer response over the KCl concentration range is that high concentrations of buffer salt might have caused the α-TTP to self-associate or dimerize only at the lipid exchange loop (in the open conformation), thus yielding similar initial fluorescence values to the determined maximum, but resulting in the transfer of much less NBD-tocopherol due to loss of the exchange loop functionality. In this case, fluorescence data would appear to be starting at or around the experimentally determined maximum but not falling to the same extent as it would if the exchange loops were fully functional, therefore yielding comparatively low transfer rates.

Under the current assay conditions, increases in KCl concentration up to 1 M actually showed a very slight increase in transfer rate of NBD-toc to acceptor LUVs, which is believed to be an artifact of the fluorescent assay being used. However, a concentration of 2 M KCl resulted in a slight decrease in transfer rate versus other concentrations. Structural alterations of the α-TTP caused by high salt concentrations might be elucidated through studies of circular dichroism (CD) and solvent-accessible surface area (SAS) data. The reduction in transfer rate at high salt concentrations, however, was believed to be possibly due to increased packing density of phospholipid head groups through their dehydration, as well as disruption of any existing electrostatic interactions between the protein and acceptor vesicle. The fact that the transfer rate was not drastically reduced over the current range of KCl concentrations suggests that transfer of NBD-toc is occurring by a collisional mechanism where the α-TTP comes into physical contact with acceptor membranes to deliver its ligand.

2.4.3 Effect of Phosphatidylserine Concentration on Transfer Rate

If a collisional transfer mechanism is suspected from the results of assays with varying acceptor vesicle and buffer salt concentration, then there are a number of secondary investigations that can be done to elucidate those specific molecular factors which influence the rate of ligand transfer. Previous studies of collisionally-dependent transport proteins have involved changes in the ionic nature of acceptor membrane phospholipids and/or their degree of unsaturation in attempting to
determine which factors influence ligand transport and to what extent. In theory, if a transport protein participates in ionic interactions with the acceptor membrane, increasing the magnitude of the charge by raising the amount of a charged phospholipid such as phosphatidylyserine (PS, Figure 27) contained in the membrane should consequently increase the transfer rate.

Figure 27: Structure of liver phosphatidylyserine. (Avanti Polar Lipids, AL, USA)

Transfer assays were performed at the standard concentration ratio of 0.5 μM NBD-toc : 5.0 μM α-TTP : 250 μM LUV, but with varying amounts of phosphatidylyserine (PS) incorporated into the acceptor vesicle population (0 %, 10 % and 25 %). Rate values for each of the % PS assays are shown in Table 4.

Table 4: NBD-toc transfer assay rate constants for increasing PS concentrations.
Transfer rates for 0 – 10 % PS were not significantly different from one another (p=0.0515 for t-test, n=10), while the difference between rates for 10 – 25 % PS did show statistical significance (p<0.05 for t-test, n=10).

<table>
<thead>
<tr>
<th>% PS</th>
<th>Rate (k, sec⁻¹)</th>
<th>Std. Error (sec⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.189</td>
<td>0.003</td>
</tr>
<tr>
<td>10</td>
<td>0.195</td>
<td>0.003</td>
</tr>
<tr>
<td>25</td>
<td>0.204</td>
<td>0.003</td>
</tr>
</tbody>
</table>

Rate constants (k₂) were similar but showed a steady increase from 0.189 ± 0.003 sec⁻¹ to 0.204 ± 0.003 sec⁻¹ between 0 % and 25 % PS loading, as shown graphically in Figure 28.
Figure 28: Forward transfer rate vs. mole % PS in acceptor LUV for α-TTP to LUV transfer assay.

Since ligand delivery by α-TTP is thought to be mediated at least in part by interactions between a positively charged area on the surface of α-TTP and anionic charges on the acceptor membrane surface (based on sequence homology with Sec14p, see Figure 7, Section 1.5.1 – The CRAL-TRIO Protein Superfamily), an increase in the amount of negatively charged phospholipid head groups was expected to result in a concurrent increase in transfer rate, as shown for other transfer proteins. There was a very slight increase in transfer rate of approximately 0.009 sec\(^{-1}\) (roughly 8 %) between 0 % PS and 25 % PS trials (0.189 ± 0.0036 sec\(^{-1}\) and 0.204 ± 0.0029 sec\(^{-1}\), respectively). Such a small rate increase over a relatively large range of PS loading values, however, rendered the change essentially negligible compared to other collisional proteins, which have shown up to 3-fold rate increases between 0 % and 10 % PS loading values, and much higher rate increases (greater than 5- to 7-fold) at up to 25 % PS loading.

The fact that transfer rate values changed little across the range of PS concentrations used in these assays indicated that under the current assay conditions, inclusion of up to 25% PS in acceptor LUV does not greatly enhance transfer activity. A possible reason for these unexpected results was that the assay conditions were not optimal. Specifically, if the standard SET buffer KCl concentration of 150 mM were sufficiently high to disrupt or interfere with the ionic interactions between α-TTP and
charged membrane lipids, any changes normally caused by increases in anionic lipid concentration might be diminished in whole or in part. Such an effect could be tested for by performing the same transfer assays described for assessing a range of % PS, but at lower KCl concentrations in both LUV and α-TTP/ligand samples (while monitoring the protein and LUV samples for stability). It is important to note, however, that reductions of effective membrane surface charge typically occur at buffer salt concentrations approaching 1 M\(^{107}\), and it is thus most likely not the case that a concentration of 0.15 M KCl would be sufficient to disrupt the electrostatic interaction between α-TTP and acceptor LUV. Thus, PS loading levels of up to 25 mole % do not seem to have a strong effect on the rate of NBD-toc transfer from α-TTP to acceptor LUV.

### 2.4.4 Effect of Cholesterol on Transfer Rate

Evidence from the assays of acceptor LUV concentration and buffer ionic strength suggests that the α-TTP may in fact be a collisional transport protein. The proposed mechanism of tocopherol transfer by α-TTP is based on the crystal structure and includes a “bulldozer” or “shovel” step utilizing a binding lid, similar to that of yeast phosphatidylinositol transfer protein Sec14p\(^{56}\), in addition to electrostatic interactions\(^{62,75}\). It is therefore plausible that changes in the physical structure of the bilayer may influence the transfer of α-toc to some degree. Cholesterol (Figure 29) is known to impart structural rigidity to membranes\(^{153}\) and can be found in vivo at levels of up to 40%\(^{154}\). Given that cholesterol is so prevalent in mammalian membranes, and that it has previously been shown to decrease the transfer rates of other collisional proteins\(^{107}\), its effect on the transfer rate of NBD-toc by α-TTP was investigated.

![Structure of cholesterol](image)

**Figure 29:** Structure of cholesterol. (Avanti Polar Lipids, AL, USA)

Transfer assays were performed at the standard component concentration ratio of 0.5 : 5 : 250 μM (NBD-toc : α-TTP : LUV, respectively), but with 0, 15 and 30 mole %
cholesterol in the acceptor LUV. Rate values for each of the cholesterol concentrations are shown in Table 5.

Table 5: NBD-toc transfer assay rate constants for increasing cholesterol concentrations. Transfer rates for 0 – 15 % cholesterol were not significantly different from one another (p=0.2143 for t-test, n=10), while the difference between rates for 15 – 30 % cholesterol also did not show statistical significance (p=0.0580 for t-test, n=10). Comparison between rate constants for 0 and 30 % cholesterol also showed no significant difference (p=0.2430).

<table>
<thead>
<tr>
<th>% Cholesterol</th>
<th>Rate (k, sec(^{-1}))</th>
<th>Std. Error (sec(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.281</td>
<td>0.025</td>
</tr>
<tr>
<td>15</td>
<td>0.266</td>
<td>0.017</td>
</tr>
<tr>
<td>30</td>
<td>0.294</td>
<td>0.018</td>
</tr>
</tbody>
</table>

Rate constants were similar at 0.281 ± 0.025 sec\(^{-1}\), 0.266 ± 0.017 sec\(^{-1}\) and 0.294 ± 0.018 sec\(^{-1}\) for concentrations of 0, 15 and 30 mole % cholesterol, respectively as shown graphically in Figure 30.

Figure 30: Forward transfer rate vs. mole % cholesterol in acceptor LUV for α-TTP to LUV transfer assay.

Since the collisional transfer mechanism of α-TTP is thought to operate in part by insertion of the protein’s lipid exchange loop into the target bilayer, and since the
presence of cholesterol in model membranes has previously been shown to decrease the transfer rates of collisional proteins \(^{107}\), we hypothesized that changes in the physical packing of lipids due to the presence of cholesterol might have an effect on transfer rate. Specifically, since cholesterol is known to influence membrane structural rigidity by associating with saturated lipids \(^{107}\), the change from a fluid state to a relatively more rigid one was investigated as a possible condition for affecting transfer rate by altering the interaction of the \(\alpha\)-TTP lipid exchange loop with the bilayer. Rate constants obtained from cholesterol loading assays were quite similar and demonstrated no overall statistically significant difference. Thus it is apparent, given these results, that inclusion of either 15 or 30 mole % cholesterol in LUVs had no effect on transfer rate.

It is possible that other membrane components may be necessary to augment the effects of cholesterol on \(\alpha\)-TTP-mediated ligand transfer, as cholesterol has been shown to interact with other membrane lipids to bring about structural changes in bilayers \(^{107,155}\). Since the investigation of cholesterol’s effect on transfer was one of many different mechanistic analyses of the \(\alpha\)-TTP, the large number and complexity of requisite control experiments associated with a thorough multi-lipid membrane structure study would have fallen outside of the main thrust of this research project, which was the development of an assay for mechanistic study. It is important to note, however, that cholesterol alone (at levels of 30 %) has indeed been shown to decrease collisional transfer rates \(^{107}\), an effect which was not seen with the current system.

The results obtained from the incorporation of cholesterol into acceptor LUV at 15 and 30 mole % exhibit no significant change in transfer rate of NBD-toc by \(\alpha\)-TTP. However, further and more detailed investigation into the nature of cholesterol’s role in affecting tocopherol transfer through its interaction with different lipid types might possibly reveal a relationship between membrane structure and transfer rate.

### 2.4.5 Effect of the Degree of Lipid Unsaturation on Transfer Rate

Since it is known that \(\alpha\)-tocopherol spontaneously associates with unsaturated and polyunsaturated lipids \(^{156-158}\), the possibility arises that \(\alpha\)-TTP may preferentially deposit tocopherol into regions rich in unsaturated lipids, and PC sources of varying acyl chain saturation were thus assessed by transfer assay. Specifically, LUVs were prepared from
samples of bovine liver mixed PC and soy PC, which have different levels of acyl chain saturation as shown in Table 6. The “S/U” designation refers to the amount of saturated vs. unsaturated lipid acyl chains in the mixed samples such that higher numbers denote lipid mixtures with greater proportions of saturated acyl chains while lower numbers indicate a greater proportion of unsaturated lipids.

It was thought that the higher level of acyl chain unsaturation in soy PC might have an effect on transfer rate compared to standard bovine mixed PC, due to a reduction in lipid packing density. With this change in membrane density, insertion of the lipid exchange loop of α-TTP into the lipid bilayer might possibly occur more favourably, thus resulting in an increase in transfer rate.

Table 6: Comparison of phospholipid acyl chain unsaturation between bovine liver mixed PC and soy mixed PC (taken from www.avantilipids.com).

Transfer assays were performed with standard LUVs made from either bovine or soy mixed PC stocks at a constant NBD-toc : α-TTP : LUV concentration ratio of 0.5 : 5 : 250 μM, respectively. Calculated rates for bovine and soy PC samples are shown in Table 7. Transfer rate constants were 0.281 ± 0.025 sec⁻¹ and 0.258 ± 0.016 sec⁻¹, respectively, as shown graphically in Figure 31.
Table 7: NBD-toc transfer assay rate constants for bovine liver and soy PC sources. Transfer rates between the two PC sources were not significantly different from one another (p=0.1294 for t-test, n=10).

<table>
<thead>
<tr>
<th>PC Source</th>
<th>Rate (k, sec(^{-1}))</th>
<th>Std. Error (sec(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine Liver</td>
<td>0.281</td>
<td>0.025</td>
</tr>
<tr>
<td>Soy</td>
<td>0.258</td>
<td>0.016</td>
</tr>
</tbody>
</table>

Figure 31: Forward transfer rate vs. PC source in acceptor LUV for \(\alpha\)-TTP to LUV transfer assay.

Since the proposed collisional transfer mechanism for \(\alpha\)-TTP is thought to occur in part by insertion of the lipid exchange loop into the bilayer \(^{62,75}\), an investigation into the effects of lipid mixtures, and specifically of differential acyl chain unsaturation, was undertaken. The rates for bovine and soy PC LUVs have substantially overlapping errors, and have no statistically significant difference between them. Furthermore, since it was apparent that no fluorescence data was lost from the raw decay curves (both sample types consistently started at fluorescence values greater than approximately 97% of the pre-determined maximum), any effects on calculated rates due to the dead time of the mixing unit having been surpassed were ruled out.

One possibility for the lack of response to the change in acyl chain unsaturation is that an increase in the number of unsaturated lipids alone is not sufficient to alter the permeability of the bilayer to the lipid exchange loop of \(\alpha\)-TTP without some sort of organizational element being present as well. Membrane lipids may need to be organized...
into microdomains through a potentially complex network of specific proteins, lipids and other membrane components, a phenomenon more sophisticated than the current in vitro studies of cholesterol and lipid source/unsaturation will allow for. Thus, the rates calculated from the current assays of differential acyl chain saturation between bovine liver and soy mixed PC indicate that there is no significant effect on the transfer rate of NBD-toc between the two PC sources.

2.4.6 Effect of Late Endosomal LUV Composition on Transfer Rate

In addition to the previously discussed assays involving changes in either the electrostatic nature or physical packing of the phospholipid bilayer, a study was done using LUVs of similar composition to that of late endosomal membranes. The late endosome is a part of the cell’s molecular sorting machinery from which it is thought tocopherol might possibly be redistributed to the plasma membrane through a process known as back-fusion \(^{144, 159, 160}\). Late endosomal membranes are known to contain 2,2'-dioleoyl-lysobisphosphatidic acid (LBPA), a unique lipid which is believed to play a role in both the degradation and transport of proteins and lipids \(^{161}\). It is also known from confocal microscopy studies that NBD-toc and α-TTP co-localize at the late endosome \(^{144}\). Thus, the possibility exists of a specific recognition event between α-TTP and LBPA which is ultimately responsible for the translocation of tocopherol from the late endosomal membrane to the plasma membrane for incorporation into very low density lipoproteins (VLDLs).

Late endosomal LUVs used in transfer assays contained the phospholipids shown in Table 8, including those with anionic head groups such as 3,3'-LBPA (Figure 32), PS, phosphatidylinositol (PI) as well as those which impart negative curvature to membranes such as phosphatidylethanolamine (PE) \(^{40}\). Due to the difficulties associated with obtaining 2,2'-dioleoyl-LBPA, including cost and stability, 3,3'-dioleoyl-LBPA was instead used in the endosomal lipid preparations.
Table 8: Phospholipid composition of late endosomal vesicles used in transfer assays.

<table>
<thead>
<tr>
<th>Lipid</th>
<th>% Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphatidylcholine</td>
<td>47</td>
</tr>
<tr>
<td>Phosphatidylethanolamine</td>
<td>20</td>
</tr>
<tr>
<td>Phosphatidylinositol</td>
<td>5</td>
</tr>
<tr>
<td>Sphingomyelin</td>
<td>5</td>
</tr>
<tr>
<td>Phosphatidylserine</td>
<td>5</td>
</tr>
<tr>
<td>Lysobisphosphatidic acid</td>
<td>15</td>
</tr>
<tr>
<td>TRITC-DHPE</td>
<td>3</td>
</tr>
</tbody>
</table>

Figure 32: Structure of 3,3'-dioleoyl-lysobisphosphatidic acid (LBPA) (Avanti Polar Lipids, AL, USA).

Transfer assays were performed using endosomal vesicles as acceptor LUVs at the standard NBD-toc : α-TTP : LUV concentration ratio of 0.5 : 5.0 : 250 μM. These assay results were compared to analogous experiments done using standard liver PC LUVs at 97% PC : 3% TRITC-PE, mole : mole. Results are displayed in Table 9, and shown graphically in Figure 33.

Table 9: NBD-toc transfer assay rate constants for standard PC and endosomal LUVs. Transfer rates between the two LUV types were significantly different from one another (p<0.0001).

<table>
<thead>
<tr>
<th>LUV Type</th>
<th>Rate (k, sec⁻¹)</th>
<th>Std. Error (sec⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard PC</td>
<td>0.189</td>
<td>0.004</td>
</tr>
<tr>
<td>Late Endosomal</td>
<td>0.231</td>
<td>0.004</td>
</tr>
</tbody>
</table>
The proposed collisional transfer mechanism of α-TTP includes two general phases, including association of the protein and bilayer through electrostatic interactions and deposition or removal of tocopherol via a "bulldozer" step (Figure 26, Section 2.4.2) in which the lipid exchange loop of α-TTP is inserted directly into the bilayer \textsuperscript{62,75}. The existence of such a complex mechanism could potentially allow for faster transfer rates when larger amounts of lipids are present which either have anionic character or are known to cause reductions in phospholipid packing density. It was thought that 3,3'-dioleoyl-LBPA might contribute to both of these phenomena by way of the negative charge on its head group and the cis-double bonds on each of its acyl chains. The calculated rate constants are in fact markedly different (p<0.0001), with the late endosomal LUV composition giving a higher transfer rate (~20 % higher value, Figure 33) at identical acceptor LUV concentrations. Thus, the current data support the general hypothesis that late endosomal lipid mixtures will contribute to an increase in transfer rate when incorporated into acceptor LUVs in this assay format.

An important point to consider at this time is the fact that the PC and PS present in the late endosomal mixtures were different than those in all other LUVs. The use of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (DOPS) instead of the analogous liver-derived lipids (liver PC and PS) was based on the fact that these lipids had been used previously as late endosomal membrane mimics. Also, since use of the late endosomal mixtures already added a high

Figure 33: Forward transfer rate vs. LUV composition for standard and late endosomal lipid mixtures in α-TTP to LUV transfer assay.
level of complexity to an otherwise fairly straightforward set of variables, this acyl chain substitution was predicted to have a minimal effect on transfer relative to the introduction of completely new lipid types.

In order to determine the contribution of the different components of the late endosomal mixture to transfer rate, each of these components would have to be assayed individually and compared to one another. Furthermore, as discussed previously (Section 2.4.4 – Effect of Cholesterol on Transfer Rate), lipids can interact with one another in specific ways, so the different interactions of varying lipid combinations within the late endosomal mixture would have to be assessed to obtain a true and thorough understanding of the individual effect of each lipid. This represents myriad possible analyses and is thus beyond the scope of the current research effort.

Although it was determined that late endosomal lipid mixtures in LUVs caused an increase in transfer rate of approximately 20 % for NBD-tocopherol transfer from α-TTP to acceptor LUV, such an increase is much lower than the expected many-fold rate enhancement which was expected using LBPA. Nonetheless, the data obtained still suggest that NBD-toc transfer to acceptor LUV occurs by a collisional mechanism, and that the presence of late endosomal lipid mixtures results in an increase in the transfer rate.

2.4.7 Effect of Buffer Viscosity on Transfer Rate

The transport of α-toc in vivo occurs within a matrix of cellular components and through a complex aqueous medium. With this in mind, a brief exploration of buffer viscosity characteristics was performed as a supplement to the aforementioned examination of buffer ionic strength. Tris-HCl buffer was compared to Sucrose/EDTA/Tris (SET) buffer (both at pH 7.4) to determine the effect, if any, of buffer viscosity on the transfer rate of NBD-toc from α-TTP to standard acceptor LUV (97% PC : 3% TRITC-PE, mole : mole). SET buffer allows the admittedly artificial assay system used herein to be at least one step closer to biological relevance by attempting to mimic the composition of the cytoplasm. Theoretically, the possibility exists for a decrease in transfer rate upon switching from Tris-HCl to the slightly more
viscous SET buffer, since the assay at its heart depends on the movement of NBD-toc - loaded protein through the buffer medium toward acceptor LUV.

Transfer assays were performed with standard liver PC LUVs at the typical ratio of 0.5 μM NBD-toc : 5.0 μM α-TTP : 250 μM LUV, but with all component stocks made up in either Tris-HCl or SET buffer. Calculated transfer rates are shown in Table 10.

**Table 10:** NBD-toc transfer assay rate constants for standard PC LUVs in SET and Tris-HCl buffer systems. Transfer rates between the two buffer systems were not significantly different from one another (p=0.1532).

<table>
<thead>
<tr>
<th>Buffer Type</th>
<th>Rate (k, sec⁻¹)</th>
<th>Std. Error (sec⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SET</td>
<td>0.279</td>
<td>0.032</td>
</tr>
<tr>
<td>Tris-HCl</td>
<td>0.305</td>
<td>0.021</td>
</tr>
</tbody>
</table>

Since the collisional transport of α-toc from α-TTP to acceptor LUVs occurs through such a complex aqueous medium as the cytosol, it is possible that the viscosity of this medium may influence the rate of ligand transfer. Specifically, an increase in viscosity could potentially slow down the dispersion of all components, reducing the frequency of collisions between the α-TTP and acceptor LUV, and thus lowering the calculated transfer rate. Conversely, a more fluid buffer system would allow for greater molecular motion and increased frequency of molecular collisions, resulting in a higher transfer rate. The difference between calculated transfer rates were shown not to be statistically significant (P=0.1532), and are represented graphically in Figure 34.
Thus, there is no apparent rate enhancement by Tris buffer in the current system and it may actually be the case that no rate enhancement exists because the viscosities of SET and Tris buffers are not sufficiently different to cause variations in the frequency of molecular interaction in solution.

2.4.8 Use of 9-AO-toc in Early Transfer Assay Development

Although the NBD-toc ligand was utilized for all described explorations of lipid composition, synthetic chemistry efforts within the research group have yielded a number of potentially useful fluorescent tocopherol analogues including a 9-anthroyloxytocopherol derivative, C9-anthroyloxy-α-tocopherol (AO-toc, Figure 35) \(^{112}\).

Figure 35: Structure of anthracene-9-carboxylic acid 9-((R)-6-hydroxy-2,5,7,8-tetramethyl-chroman-2-yl]-nonyl ester, or AO-tocopherol.

This ligand was the first of the collection of analogues to be synthesized and characterized, and was employed during the initial stages of tocopherol transfer assay
design due to the extensive use of anthroyloxy-labeled ligands in previous lipid transfer studies. Concurrent with the ligand binding studies of the AO-toc with α-TTP, the full range of background analyses was performed in a lipid environment as described for NBD-toc. Following this, the AO-toc ligand was used along with N-(7-nitro-2,1,3-benzoazadiazol-4-yl) phosphatidylethanolamine (NBD-PE, Figure 36) as a quenching FRET partner in stopped-flow transfer assays.

Figure 36: Structure of 1,2-Dipalmitoyl-sn-Glycero-3-Phosphoethanolamine-N-(7-nitro-2,1,3-benzoazadiazol-4-yl) (Avanti Polar Lipids, AL).

The performance of AO-toc, however, was inconsistent and results from transfer assays were often drastically different. While some sample injections resulted in “clean” or “regular” looking fluorescence decay curves (as seen later with NBD-toc), others resulted in either a very shallow decay curve in the middle of multiple “clean” injections or an anomalous “hump” in the early time points (Figure 37).

Figure 37: Sample fluorescence decay curves for AO-toc transfer assay between α-TTP and PC/NBD-PE LUVs at 0.375 μM : 3.75 μM : 675 μM (AO-toc : α-TTP : LUV, respectively). Anomalies encountered during the early stages of assay development included A) Shallow decay curves relative to the others within a repetition set (pink and purple, respectively) and B) The occurrence of a “hump”, or a brief rise and fall in fluorescence, shortly after sample injection.
Since reproducible results were not obtained from transfer assays with AO-toc and α-TTP to acceptor LUV, further analysis of transfer including calculation of rate constants was not performed. The poor behaviour of AO-toc in solution is believed to be a result of both its poor solubility and relatively high susceptibility to photobleaching (up to ~ 20 % of the signal over the duration of a transfer assay). Also, some degree of nonspecific binding to α-TTP was seen \(^95,112\). At roughly the same time as difficulties were being experienced with the AO-toc ligand, the NBD-toc ligand was synthesized and its binding to α-TTP characterized \(^95,112\). Upon further analysis, not only was the NBD-toc ligand found to have a better \(K_d\) than AO-toc (56 ± 15 nM vs. 279 ± 124 nM, respectively) \(^112\), but the TRITC-PE FRET partner was found to have a better quenching capacity for NBD-toc than the NBD-PE FRET partner did for AO-toc (Figure 38). In fact, as mentioned earlier the TRITC-PE quencher consistently eliminated greater than 75 % of the fluorescence signal from NBD-toc over a range of NBD-toc concentrations, whereas the NBD-PE quencher only accounted for a quenching factor of approximately 50% at working concentrations.

![Figure 38](image)

**Figure 38:** Quenching capacity of FRET quenchers for their respective tocopherol analogue fluorophore partners over a range of donor fluorophore concentrations. Graphs shown indicate donor ligand fluorescence intensity in the presence (dashed lines) and absence (solid lines) of quencher for A) NBD-toc with TRITC-PE and B) AO-toc with NBD-PE.

The desirable characteristics of this newly available ligand including its lower \(K_d\), better FRET interaction with its respective quencher partner, and better behaviour than AO-toc under assay solution conditions led to its use in all further transfer rate investigations of the α-TTP.
3 CONCLUSION AND FUTURE PERSPECTIVES

A FRET-based assay has been developed for assessment of the rate of ligand transfer between α-TTP and acceptor lipid membranes under varying conditions. The assay is based on proven techniques such as large unilamellar vesicle preparation via extrusion and FRET monitoring, and follows the signal decay as the NBD-tocopherol analogue is transferred from α-TTP (high fluorescence) to quenching vesicle (low fluorescence).

The mechanism of ligand transfer by α-TTP was investigated by varying the concentration and composition of acceptor LUVs, as well as by varying the ionic strength and viscosity of the buffer medium. Increasing the concentration of acceptor lipid relative to the NBD-toc/α-TTP complex resulted in a simultaneous rise in transfer rate such that a 6-fold excess of acceptor LUV caused a roughly 2.6-fold rate increase. This was the first piece of evidence that indicated a collisional transfer mechanism for α-TTP. Additional support for a collisional mechanism was found in the fact that transfer rates increased in the presence of both PS (5 % rate increase between 10 and 25 % PS) and late endosomal membrane preparations (20 % faster than standard PC/TRITC-PE vesicles), and were not abolished by the introduction of high salt concentrations in the buffer (up to 2 M KCl). While the general insensitivity to salt concentration was a strong indicator of collisional transfer, the aforementioned rate increases in the presence of PS and late endosomal preparations were, however, relatively small compared to the multiple-fold increases seen in analogous systems for other ligand/protein partners\(^{107,150}\).

In contrast to the above results, assays involving cholesterol, acyl chain unsaturation and buffer viscosity did not affect transfer rates by any significant level. The apparent lack of rate response for some conditions was initially suspected to be due to the truncation of some fraction of early data points by overrunning the dead time of the stopped-flow unit (as explained in detail in Section 2.4 - NBD-toc / h-α-TTP Transfer Assays – Investigations). However, as described previously, the average rate increase of roughly 10 % obtained from simulating the lost data was still not of a sufficient magnitude to make up for the expected multiple-fold rate increases seen in other
fluorescent ligand transfer assay systems. Still, a further, more comprehensive investigation of the effects of cholesterol, acyl chain unsaturation and buffer viscosity may indeed yield statistically significant rate changes compared to standard bovine PC/TRITC-PE LUV rates.

Evidence suggests that the mechanism of NBD-toc transfer by α-TTP is collisional, but that it is possibly not regulated by electrostatic forces to the degree previously thought. Instead, the lower than expected rate results of phosphatidylserine (PS) assays seem to suggest that hydrophobic forces may play also play a significant role in the protein/membrane interaction process. A dedicated and broad survey of lipids might, however, uncover a specific charged molecule (or combination of molecules) responsible for enhancing the rate of tocopherol transfer by α-TTP.

Originally, a different tocopherol analogue (9-AO-toc) was characterized and used in transfer assays. However, problems with solubility, photobleaching and nonspecific binding to protein made the ligand very difficult to work with in aqueous solution, and the NBD-toc ligand was thus chosen for all future investigations.

The FRET-based solution assay is complemented by a new technology known as dual polarization interferometry (DPI) 162. This is a continuous flow system which can be used to study protein-membrane interactions, generating data pertaining to the thickness, mass and density of bilayers deposited on a sample chip under different conditions 163. In tandem with the current assay style, this mode of analysis could be used to study the interaction of α-TTP with various membrane types. It should be noted that the FRET-based solution assay is still necessary to examine such processes as tocopherol delivery to membranes, the effect of SUV and highly curved membranes on transfer, and intervesicular transfer.

This work has provided a reliable assay for monitoring the transfer of fluorescent tocopherol analogues by α-TTP. It has also provided strong evidence for the existence of a collisional transfer mechanism, however the nature of the forces governing the interaction between α-TTP and acceptor membranes is still somewhat unclear. Specifically, the extent to which both electrostatic and hydrophobic forces influence ligand transfer must now be investigated in greater depth. This might be done through the testing of various anionic lipids as well as lipids that impart different curvature
stresses to bilayers or have different packing characteristics within bilayers. Another possibility is to create point mutants of α-TTP to change the electrostatic or hydrophobic nature of the protein itself in order to determine which residues are important for membrane interaction. With this information at hand, it may be possible to obtain a clearer understanding of the molecular events surrounding the α-TTP-mediated transfer of tocopherol between intracellular membranes, and in a broader sense, the fate of vitamin E in vivo.
4 MATERIALS AND METHODS

4.1 Reagents and Stock Solutions

4.1.1 Solutions for Bacterial Cell Culture and Protein Expression

The following is a list of all stock solutions used for bacterial cell culture and protein expression procedures:

Luria-Bertani Broth – 5 g/L NaCl, 5 g/L yeast extract, 10 g/L tryptone (all from BioShop, Burlington, ON) dissolved in distilled water. Autoclaved at 121°C for 15 minutes.

Chloramphenicol – 20 mg/mL chloramphenicol (BioShop, Burlington, ON) in EtOH, stored at -30°C.

Kanamycin – 30 mg/mL kanamycin (BioShop, Burlington, ON) in 18 ohm MilliQ water, stored at -30°C.

Isopropyl-d-thiogalactoside (IPTG) – 1.0 M solution in distilled water, stored at -30°C (BioShop, Burlington, ON).

4.1.2 Solutions for Protein Isolation and Metal Affinity Chromatography

NiSO₄·6H₂O (0.1 M) – a 0.1 M solution in MilliQ water (Aldrich).

Mono-PO₄³⁻ (0.25 M) – 5.998 g mono-phosphate dissolved in 200 mL distilled water (BioShop, Burlington, ON).

Bis-PO₄³⁻ (0.25 M) – 7.098 g bis-phosphate dissolved in 200 mL distilled water (BioShop, Burlington, ON).

Imidazole (4 M) – 2.72 g in 10 mL distilled water (BioShop, Burlington, ON).

10X Start Buffer (0.25 M phosphate, 2.5 M NaCl, pH 7.4) – 0.25 M mono-phosphate and 0.25 M bis-phosphate mixed to pH 7.5 to make 10X phosphate solution, then add 18.9993 g NaCl with 130 mL of 10X phosphate solution.

1X Column Binding Buffer (40 mM imidazole, 25 mM PO₄³⁻, 0.25 M NaCl, 40 mM imidazole, pH 7.4) – 10 mL of 10X start buffer, 1 mL 4 M imidazole diluted to 100 mL distilled water.

100 mM Imidazole Wash Buffer – 10 mL of 10X start buffer, 2.5 mL of 4.0 M imidazole, pH adjusted to 7.4, diluted to 100 mL with distilled water.
200 mM Imidazole Wash Buffer – 10 mL of 10X start buffer, 5.0 mL of 4.0 M imidazole, pH adjusted to 7.4, diluted to 100 mL with distilled water.

400 mM Imidazole Elution Buffer - 10 mL of 10X start buffer, 10 mL of 4.0 M imidazole, pH adjusted to 7.4, diluted to 100 mL with distilled water.

SET Buffer – 250 mM Sucrose, 1 mM EDTA, 50 mM Tris, 100 mM KCl, pH 7.4.

4.1.3 Solutions for SDS-PAGE Electrophoresis

Stacking Buffer (0.5 M) – 30.28 g Tris base (BioShop, Burlington, ON) dissolved in 200 mL distilled water, pH adjusted to 6.8 and diluted to 500 mL with distilled water.

Separating Buffer (1.5 M) – 90.83 g Tris base (BioShop, Burlington, ON) dissolved in 300 mL distilled water, pH adjusted to 8.8 and diluted to 500 mL with distilled water.

10X Loading Buffer – 0.1 M Na₂EDTA (BioShop, Burlington), pH 8.0, 1.0% w/v SDS (Sigma, Oakville, ON), 0.25% w/v bromophenol blue (Aldrich).

10X Running Buffer – 0.25 M Tris, 2.0 M glycine, pH unadjusted (Sigma, Oakville, ON).

1X Running Buffer – 100 mL of 10X running buffer and 10 mL 10% w/v SDS into 890 mL distilled water.

SDS-PAGE Gel Fixing Solution – 40% ethanol, 10% acetic acid in distilled water.

Coomassie Brilliant Blue (CBB) Gel Staining Solution – 1.0 g/L CBB R-250 (Bio-Rad, Hercules, CA), 40% MeOH, 10% acetic acid in distilled water.

SDS-PAGE Gel Destaining Solution – 10% MeOH, 10% acetic acid in distilled water.

4.2 Bacterial Cell Culture and Protein Expression

A 10 mL sample of LB was inoculated from a freeze culture of E. coli BL21(DE3) cells containing the pET 28/α-TTP construct, after the addition of 10 µL chloramphenicol and 10 µL kanamycin stock solutions. This preculture sample was incubated overnight at 37°C and used to inoculate pre-warmed culture flasks of 150 mL LB broth on a 1:100 (inoculant:broth) volume scale. The culture flasks were secured and left in a shaker/incubator at 180 rpm and 30°C until they reached an OD₆₀₀ of 0.4 - 0.6 (typically closer to 0.6), at which time IPTG was added at a final concentration of 1 mM to induce protein expression. The expression period was allowed to proceed for another
10-14 hrs. At this time, the contents of two flasks were typically transferred to one pre-weighted centrifuge tube and centrifuged for 12 min. at 12,000 rpm and 4°C. The supernatant was decanted and disposed of as waste, while the remaining pellet was weighed, flash frozen in an ethanol bath and stored at -80°C until use.

4.3 Protein Isolation

The cell pellet was removed from the ethanol bath in the -80°C freezer and a series of 2-3 freeze-thaw steps was performed, each time waiting until the pellet was completely thawed before reintroduction into the ethanol bath. Generally, complete thawing could be expedited by manually breaking up a partially-thawed pellet into a few smaller pieces with a sterile stainless steel spatula. After the final freeze-thaw step, the thawed pellet was suspended in approximately 6 mL of 1X column binding buffer (for every ~3 g cell mass used) and transferred to a 100 mL centrifuge tube. Cell pellet remnants were washed from the freezer storage vessel with another 2 mL of 1X column binding buffer, added to the original suspension and placed on ice. The sample was then subjected to three to four rounds of sonication at 50% output (~12 Watts), each round including 45 pulses lasting one second, with a one second pause between pulses. Between rounds of sonication, the sample was given adequate time to cool down (~1-2 min.). Next, the sample was centrifuged at 14,000 rpm for 14 min. at 4°C to remove any contaminant cell debris particles from the supernatant. Supernatant containing the soluble protein was decanted and kept on ice while the purification column was prepared.

4.4 Protein Purification / Metal Chelate Affinity Chromatography

A prepacked 1 mL HiTrap™ affinity column (G.E. Healthcare Biosciences, Uppsala, Sweden) was washed with 5 mL milliQ water to remove ethanol from storage. The column was charged by flowing 1 mL 0.1 M NiSO₄•6H₂O through, followed by another 5 mL milliQ water. The column was then equilibrated by washing with 5 mL 1X column binding buffer (all flow rates were 1-4 mL/min. as detailed in the HiTrap manual). The supernatant obtained from the cell lysis step was sterile-filtered with 0.45 μm-pore diameter Durapore® membrane filters (Millipore) and then applied to the column at a flow rate of 1-2 mL/min. The column was then rinsed with 5 mL of 1X
column binding buffer. This was followed by washes of 100 mM- and 200 mM imidazole wash buffer at 5 mL each, maintaining a 1-3 mL/min. flow rate. Following these wash steps, the protein was eluted from the column with a single rinse of 5 mL 400 mM imidazole elution buffer, collecting approximately 500 uL in each of 10 fractions.

4.5 Protein Quantitation and Characterization

4.5.1 Bradford Assay

This reagent and method were utilized after each protein purification procedure to quantify the eluted protein fractions. A calibration curve was created for each new bottle of Bradford reagent (BioShop, Burlington, ON). Usually, six standards of bovine serum albumin (BSA) (Sigma, Oakville, ON) ranging from 25 – 500 μg/mL were prepared from either a 2 mg/mL BSA stock solution or a lyophilized BSA solid stock bottle. All samples were diluted with 400 mM imidazole elution buffer from the purification column step. Standards were made up by adding 50 μL of standard solution to 1 mL of Bradford reagent, with a blank consisting of 50 uL milliQ water in place of protein sample, followed by a 5-15 min. incubation at room temperature, then a measurement of the absorbance at 595 nm. Typically, this curve was performed in duplicate or triplicate and the averages of all absorbance values plotted as the standard curve for protein concentration determination.

4.5.2 SDS-PAGE Analysis

Purified protein samples were characterized via SDS-PAGE, for which all solutions and reagents were prepared according to the recipes in Table 11. After degassing of the gel mixtures by aspiration (approximately 5 – 10 min.), the N,N,N',N'-tetramethylethylenediamine (TEMED) and ammonium persulfate (APS) were added to the resolving gel mixture, and the solution transferred to a pre-assembled cartridge in the casting unit until it was approximately ¾ full. A layer of isopropanol was gently applied to the solution surface and the solution allowed to polymerize for 25 – 45 min., at which time the isopropanol was carefully and thoroughly removed by washing with distilled water and the residual water blotted away with filter paper. Next, the stacking gel mixture was treated with TEMED and APS and carefully layered on top of the resolving
gel. The well casting comb was inserted and the gel was left until polymerized, usually being stored overnight at 4°C to ensure complete polymerization. SDS-PAGE gels were run at 100 V for approximately two hours. Completed gels were placed in a fixing solution for approximately one hour and then rocked overnight in a CBB staining solution, prior to destaining until the protein bands were clearly distinguishable.

Table 11: Gel recipes for SDS-PAGE analysis of protein samples.

Resolving Gel (15 % acrylamide)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>MilliQ water</td>
<td>4.51 mL</td>
</tr>
<tr>
<td>40 % acrylamide/bis-acrylamide (37.5:1)</td>
<td>4.5 mL</td>
</tr>
<tr>
<td>Resolving Gel Buffer (1.5 M Tris-HCL, pH 8.8)</td>
<td>3.0 mL</td>
</tr>
<tr>
<td>10 % SDS</td>
<td>120 μL</td>
</tr>
<tr>
<td>50 % glycerol</td>
<td>24 μL</td>
</tr>
<tr>
<td>TEMED</td>
<td>5 μL</td>
</tr>
<tr>
<td>10 % APS</td>
<td>90 μL</td>
</tr>
</tbody>
</table>

Stacking Gel (5.5 % acrylamide)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>MilliQ water</td>
<td>2.51 mL</td>
</tr>
<tr>
<td>40 % acrylamide/bis-acrylamide (37.5:1)</td>
<td>0.57 mL</td>
</tr>
<tr>
<td>Stacking Gel Buffer (0.5 M Tris-HCL, pH 6.8)</td>
<td>1.00 mL</td>
</tr>
<tr>
<td>10 % SDS</td>
<td>20 μL</td>
</tr>
<tr>
<td>50 % glycerol</td>
<td>8 μL</td>
</tr>
<tr>
<td>TEMED</td>
<td>5 μL</td>
</tr>
<tr>
<td>10 % APS</td>
<td>25 μL</td>
</tr>
</tbody>
</table>

4.6 LUV Suspension Preparation

4.6.1 General LUV Preparation Method

The desired volumes of stock lipids in chloroform and NBD-tocopherol in EtOH were transferred to an 8 mL glass sample vial. The contents were placed under a stream of Ar gas until the bulk of the liquid solvent was removed, then transferred to a high-vacuum apparatus for 4 – 6 hours to ensure complete solvent removal. If the lipids were not to be used immediately, the vials were then flushed with Ar gas, wrapped with
none
Parafilm® and stored at 4°C overnight. Otherwise, the appropriate volume of SET buffer was added to each vial to give the desired final concentration of total lipid and the vials were agitated until all lipid film had been removed from the inner walls (usually 5 – 10 min.). The suspended lipid contents were then left to completely hydrate for 20 – 30 min. at room temperature, resulting in the creation of multi-lamellar vesicles (MLVs).

During this time, the LiposoFast extruder (Avestin, Ottawa) was rinsed with milliQ water and the integrity of all O-rings, screens and seals was verified prior to assembly and insertion of a 100 nm membrane. Specifically, the stainless steel casing was disassembled and one polycarbonate housing placed face-up into one end of the casing. This was held vertically and level in one hand, while the membrane was centered horizontally on the O-ring and the middle barrel of the casing slid overtop and screwed into the first casing end. Care was taken to ensure that the membrane did not become unseated from the O-ring due to force or static electricity. Next, the second polycarbonate housing unit was carefully lowered into the casing barrel to rest on the membrane (to “sandwich” the membrane between both O-rings) and the other end of the stainless steel casing was screwed onto the middle casing barrel to hand-tightness. The 1 mL acceptor syringe barrel with plunger was attached to one side of the unit and left at the “in” position, while the empty donor syringe barrel was attached to the other side. This donor barrel was filled with approximately 1 mL of MLV suspension and the plunger inserted. Between 11 and 13 passes were made of the lipid suspension through the extrusion membrane, with the total number always being odd and the process ending in the acceptor syringe to ensure that there was no MLV contamination of the final LUV sample. At the end of each extrusion process, the acceptor syringe was disconnected and emptied into a clean glass sample vial. Following all extrusion runs, the LiposoFast unit and syringes were rinsed twice with 2 M KOH followed by at least five rinses of distilled water. Typically, the O-rings and screens were removed and cleaned separately with distilled water and Kimwipes® (Kimberly-Clark Inc., Mississauga).

4.6.2 PC LUVs

LUVs composed of 100% liver-α-phosphatidylcholine (PC) (Avanti Polar Lipids, Alabaster, AL) were made by the method described in Section 4.6.1.
4.6.3 PC / TRITC-PE LUVs

LUVs composed of liver-α-phosphatidylcholine (PC) (Avanti Polar Lipids, Alabaster, AL) and N-(6-tetramethylrhodaminethiocarbamoyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt (TRITC DHPE) (Invitrogen / Molecular Probes, Burlington) were made by the method described in Section 4.6.1. The final amount of TRITC-PE was either 1, 3 or 5 mole %.

4.6.4 PC / PS / TRITC-PE LUVs

LUVs composed of PC, liver-α-phosphatidylserine (PS) (Avanti Polar Lipids, Alabaster, AL) and TRITC-PE were made by the method described in Section 4.6.1. The final amount of TRITC-PE was either 1 or 3 mole % and the final amounts of PS were 5, 10 and 25 mole %.

4.6.5 PC / Chl / TRITC-PE LUVs

LUVs composed of PC, cholesterol (Chl) (Avanti Polar Lipids, Alabaster, AL) and TRITC-PE were made by the method described in Section 4.6.1. The final amount of TRITC-PE was either 1 or 3 mole % and the final amounts of cholesterol were 15 and 30 mole %.

4.6.6 Soy PC / TRITC-PE LUVs

LUVs composed of soy phosphatidylcholine (Soy PC) (Avanti Polar Lipids, Alabaster, AL) and TRITC-PE were made by the method described in Section 4.6.1. The final amount of TRITC-PE was 3 mole %.

4.6.7 Late Endosomal LUVs

LUVs composed of late endosomal mixture of lipids were prepared as described in Section 4.6.1. These LUVs were mixtures of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), sphingomyelin (SM, brain), 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (DOPS), L-α-phosphatidylinositol (PI, liver), lysobisphosphatidic acid (LBPA) and TRITC-DHPE. The final amount of TRITC-DHPE was 3 mole %.
4.7 General Fluorescence Measurements

4.7.1 NBD-tocopherol Characterization and Fluorescence Signal Optimization

Using the Quantamaster 4 fluorescence spectrophotometer (PTI, New Jersey) and a Hi-Tech SFA-20 Stopped Flow apparatus (Bradford on Avon, UK), a synchronous scan of NBD-toc was performed to determine the excitation and emission maxima. An aliquot of the NBD-toc stock solution was diluted with EtOH to a concentration of 1 μM and transferred to a 600 μL quartz microcuvette (Hellma, Müllheim, Germany). The excitation and emission maxima were then used to optimize such physical parameters as slit width and lamp intensity, while also allowing for the determination of environmental effects and ideal ligand concentrations for acceptable signal-to-noise ratios.

4.7.2 Environmental Effects of NBD-tocopherol Fluorescence

Samples of NBD-toc in both PC LUV and α-TTP were prepared to assess any impact of the immediate environment on ligand fluorescence intensity and wavelength maxima. An aliquot of NBD-toc stock solution in EtOH was added to a suspension of 100% PC LUV (lacking quencher) (final concentrations of 0.125 μM and 125 μM, respectively) and a synchronous scan was done. Subsequent scans were performed to ensure full incorporation of NBD-toc into the LUVs through a stabilized fluorescence signal, and a fluorescence maximum value was determined. An assessment of NBD-toc fluorescence in α-TTP was also done by adding an aliquot of NBD-toc stock solution to a solution of α-TTP (final concentrations of 0.125 μM and 1.25 μM, respectively) and performing a synchronous scan. Subsequent scans were again performed to ensure full incorporation of ligand and a fluorescence maximum value was determined.

4.8 Relative Partition Coefficient (K_p) Determination

To maintain unidirectional transfer in the current assays while ensuring minimal data loss due to component ratio issues, it was necessary to determine the partition coefficient (K_p) for NBD-toc between α-TTP and acceptor LUV. This was done by measuring the stabilized fluorescence signal of NBD-toc (λ_ex = 469 nm, λ_em = 526 nm) at a given molar ratio of α-TTP : LUV after adding PC / TRITC-PE LUVs into a solution containing 2.5 μM α-TTP and 0.25 μM NBD-toc in SET buffer at pH 7.4. Briefly, a 1.4
mL aliquot of NBD-toc/α-TTP stock solution (0.25 uM and 2.5 uM, respectively) was placed into a 3 mL cuvette, and 1.4 mL of SET buffer was added and mixed to serve as a background. All LUV concentrations in the K_p range involved a similar mixing scheme and the use of increasing concentrations of LUV added through decreasing dilution factors of the stock LUV solution. The decrease in NBD-toc fluorescence upon mixing of NBD-toc-containing α-TTP with quenching LUVs was related to K_p by the following equation,

\[
1 / \Delta F = 1 / K_p (1 / \Delta F_{\text{max}}) ([\text{LUV}] / [\text{TTP}]) + 1 / \Delta F_{\text{max}} \quad \text{(Eq. 1)}
\]

where \( \Delta F \) is the difference between the initial fluorescence of NBD-toc in α-TTP and the fluorescence at a given protein/LUV ratio, and \( \Delta F_{\text{max}} \) is the maximum difference in NBD-toc fluorescence \(^{101}\). A range of LUV concentrations from 125 to 5000 uM were tested, and the fluorescence values from each trial plotted together. To obtain a value for K_p, a plot of \( 1 / \Delta F \) versus \( (1 / \Delta F_{\text{max}}) ([\text{LUV}] / [\text{TTP}] \) was constructed, where the slope was equal to \( 1 / K_p \) \(^{148}\).

### 4.9 Quenching Capacity of TRITC-PE

To determine the quenching capacity of TRITC-PE, samples of 100% PC LUVs as well as TRITC-PE LUVs were made at equal concentrations (each made at both 125 uM and 250 uM) and their fluorescence spectra acquired for use as background measurements. Aliquots of NBD-toc stock solution in ethanol were then added to each LUV sample to give equal final concentrations of 0.25 uM and measured (\( \lambda_{\text{ex}} = 469 \) nm, \( \lambda_{\text{em}} = 526 \) nm) until signal stabilization. The intensities of each sample were compared to the background measurements obtained prior to addition of NBD-toc to assess the quenching capacity of TRITC-PE. Background data were also used to compare the contribution of this quencher, if any, to assay fluorescence signal due to overlap of emission spectra with NBD-toc.

### 4.10 Signal-to-Noise Determination

Simply measuring the fluorescence emission intensity of NBD-toc / α-TTP at different concentrations can provide an idea of the fluorescence magnitude vs.
concentration, but signal-to-noise values must be determined under assay conditions (i.e. — when mixed with acceptor LUV). Different overall concentrations of ligand/protein solution (1 : 10, NBD-toc : α-TTP) and acceptor LUV solution (always at 50-fold α-TTP concentration) were injected and mixed, and their signal-to-noise ratios analyzed. This was done by dividing the noise value (the average magnitude of signal fluctuation in the transfer curve) by the overall signal intensity. Overall concentrations were 0.25 uM NBD-toc : 2.5 uM α-TTP : 125 uM LUV, and 0.5 uM NBD-toc : 5.0 uM α-TTP : 250 uM LUV. Noise values under 5 % were considered acceptable.

4.11 Photobleaching Determination

To determine the degree of NBD-tocopherol photobleaching in α-TTP and in LUVs, two different samples were analyzed. The first was a sample of NBD-tocopherol incubated with α-TTP at a 1:10 ratio for 15-25 min. and then injected (mixed) with an equal volume of SET buffer. This sample preparation was similar to that used in determining the NBD-tocopherol / α-TTP fluorescence maximum at the start of a given set of assays, and the two determinations could actually be made using the same sample. Once enough sample was injected using the stopped-flow device to reach a stabilized fluorescence signal (~1.0 mL sample + 200-400 uL milliQ water to push through), it was left under continuous illumination for the duration of a transfer assay. The difference between the initial and final signal intensities was expressed as a fraction of the total initial signal and was equal to the percent of initial signal loss due to photobleaching of fluorophore in protein. The second type of sample was a solution of PC / TRITC-PE LUVs to which NBD-tocopherol was added exogenously. After the fluorescence signal stabilized (i.e. — all NBD-tocopherol had entered the LUV bilayer), the sample was left under continuous illumination for the duration of a transfer assay and the fluorescence decrease monitored. Again, the difference between the initial and final signal intensities was expressed as a fraction of the total initial signal and was equal to the percent loss of initial signal due to photobleaching in acceptor LUV.
4.12 The α-TTP - to - LUV Transfer Assay with NBD-tocopherol

4.12.1 α-TTP/NBD-tocopherol and LUV Sample Preparation

Four to five sample injections with the stopped-flow apparatus were typically made (totaling 800 - 1000 µL) before following with water, as this allowed for between three and four non-diluted injection runs (i.e. - transfer events) to be monitored from each 1 mL of sample. This required a partial reloading of each sample syringe after three injections. Also, calculations for α-TTP / NBD-tocopherol incubation mixtures and acceptor vesicle dilutions were made to give 1.2 mL of sample per assay to account for the small volume of sample lost on the walls and under the caps of sample vessels and in the loading syringes. The required volume of α-TTP solution was added to the appropriate amount of SET buffer in a glass sample vial, followed by addition of NBD-tocopherol to give a ratio of NBD-tocopherol : α-TTP of 1:10. NBD-tocopherol was added last for two reasons; First, to avoid denaturation, the α-TTP could not be subjected to an overall EtOH concentration of more than ~1-1.5 % (v/v), and second, because the aliquot of NBD-tocopherol in EtOH was usually less than 20 µL, the solvent would evaporate very quickly if added onto the dry vial walls, potentially causing NBD-toc solubility problems. Enough α-TTP/NBD-tocopherol solution was made to allow for all planned repetitions and assays plus a control run of α-TTP/NBD-tocopherol with buffer (to determine the theoretical maximum fluorescence value) to ensure that all assays performed could be compared to the theoretical maximum starting signal. This stock was incubated for 15-25 min. At the same time, LUV samples were made by diluting aliquots of stock LUV suspensions with appropriate volumes of SET buffer.

4.12.2 Stopped-Flow System Preparation and Sample Injection

The stopped-flow system (Figure 39) was rinsed with 2-3 mL milliQ water and baseline signal verified (typically equal to or less than ~1500 arbitrary units).
The importance of continuous learning and the development of new skills cannot be overstated in today's rapidly changing world. Adapting to new technologies and methodologies is crucial for individuals and organizations alike. By staying informed and actively seeking opportunities to learn, we can ensure our skills remain relevant and our expertise continues to grow. This ongoing commitment to education and professional development is essential for personal and professional success in the 21st century.
Following rinsing, the instrument was primed by loading each injection syringe with the appropriate sample and making a total of four injections (as described in Section 4.12.1). A 1 mL aliquot of milliQ water was then pushed through the lines, but after each injection the fluorescence signal was monitored until the acquisition program end time. This was continued until the sample had been flushed from the lines, at which time an additional 2 mL of milliQ water was used to flush the system. A rinse of ~2 mL MeOH followed to remove any organic components remaining in the lines, syringes or sample chamber, and this was washed out by a final series of milliQ water injections totaling 5 mL. All experiments were performed at ambient temperature, or 19°C.

4.12.3 Data Analysis

The PTI Quantamaster 4 fluorescence spectrophotometer was used to acquire all raw data from assays, which was subsequently exported to Excel (Microsoft, Redmond, WA). The raw data were in units of either Fluorescence Intensity (a.u.) vs. Wavelength (nm) (for characterization and optimization studies) or Fluorescence Intensity (a.u.) vs. Time (s) (for all transfer assays).

Once in Excel, the raw time data were normalized by subtracting the actual injection time (this was never exactly at time zero) from all subsequent values in an injection run, or trace. This ensured that traces started at exactly t = 0 seconds. Next, the

Figure 39: Representative diagram of sample lines in the SFA-20™ stopped-flow mixing device (TgK Scientific, Bradford on Avon, UK).
final fluorescence data point in each trace was subtracted from each previous data point in the trace to give a normalized range (fluorescence values that will end at zero), and then each of these values was divided by the normalized fluorescence intensity at time zero (the initial intensity upon injection). The overall result was that for graphing and calculation purposes, time values started at zero and increased as the assays progressed, and fluorescence values started at a relative intensity of one and decreased to zero. All interpreted graphs and calculations would thus be representations of how quickly each assay condition reached its endpoint; and all endpoints, unless otherwise noted, were similar.

Rate determinations were made by graphing relative fluorescence intensity (a.u.) vs. time (s) for each assay repetition and fitting data to a two-phase exponential decay curve model using the equation: \( Y = \text{Span1}*\exp(-K1*X) + \text{Span2}*\exp(-K2*X) + \text{Plateau}; \)
where \( Y \) = relative fluorescence intensity at a given time, \( K1 \) = rate constant for the slow process, \( K2 \) = rate constant for the fast process and \( X \) = time. It should be noted here that when prompted to compare the quality of fit between one- and two-phase exponential decay models, Prism preferred the fit of a two-phase model with all data sets (see residuals plots, Section 2.4). The slow process (represented by \( K1 \) in the equation) is presumed to be a small degree of photobleaching of the ligand, while the fast process (represented by \( K2 \)) is most likely the \( \alpha \)-TTP-mediated delivery of NBD-toc to LUVs.

4.13 Equipment

Corning pH Meter 445 (Corning)
Shaker Incubator (Gallenkamp)
Sorvall RC 5C Plus (SLA-3000 and SS-34 rotors) (Mandel Scientific)
Sonic Dismembrator Model 100 (Fisher Scientific)
Sorvall MC 12 Microcentrifuge (Mandel Scientific)
Mini Protean II (Bio-Rad Laboratories, Hercules, CA)
LiposoFast Extruder Model LF-101 (Avestin, Inc., Ottawa)
Quantamaster 4 fluorescence spectrophotometer with Felix 32 analysis software (PTI, New Jersey)
Hi-Tech SFA-20 Stopped Flow apparatus (Bradford on Avon, UK)
Spectronic Genesys 2 spectrophotometer (Spectronic Instruments)
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Appendix I – Example of Truncated Initial Time Point Data.

A) Early data is apparently missing from the acquisition set.

B) Close up view of the truncated early data points.
Appendix II – Simulated Initial Time Point Data Inserted Into Fluorescence Decay Curve.

A) Early data has been simulated by extrapolation and inserted.

B) Close up of simulated early data points.