Ligand Specificity in the CRAL-TRIO Protein Family

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Received January 16, 2003

ABSTRACT: Intracellular trafficking of hydrophobic ligands is often mediated by specific binding proteins. The CRAL-TRIO motif is common to several lipid binding proteins including the cellular retinaldehyde binding protein (CRALBP), the α-tocopherol transfer protein (α-TTP), yeast phosphatidylinositol transfer protein (Sec14p), and supernatant protein factor (SPF). To examine the ligand specificity of these proteins, we measured their affinity toward a variety of hydrophobic ligands using a competitive [^{3}H]-*RRR*-α-tocopherol binding assay. α-TTP preferentially bound *RRR*-α-tocopherol over all other tocols assayed, exhibiting a K_d of 25 nM. Binding affinities of other tocols for αTTP closely paralleled their ability to inhibit in vitro intermembrane transfer and their potency in biological assays. All other homologous proteins studied bound α-tocopherol but with pronouncedly weaker (> 10-fold) affinities than α-TTP. Sec14p demonstrated a K_d of 373 nM for α-tocopherol, similar to that for its native ligand, phosphatidylinositol (381 nM). Human SPF had the highest affinity for phosphatidylinositol (216 nM) and γ-tocopherol (268 nM) and significantly weaker affinity for α-tocopherol (K_d 615 nM). SPF bound [^{3}H]-squalene more weakly (879 nM) than the other ligands. Our data suggest that of all known CRAL-TRIO proteins, only αTTP is likely to serve as the physiological mediator of α-tocopherol's biological activity. Further, ligand promiscuity observed within this family suggests that caution should be exercised when suggesting protein function(s) from measurements utilizing a single ligand.

Due to the thermodynamic barriers imposed on diffusion of a hydrophobic molecule through an aqueous environment, specific binding proteins exist that may facilitate lipid transport. The transfer protein must serve a dual purpose: it must be able to recognize a specific lipid with high affinity, and it must also be able to release the ligand to the acceptor membrane. Depending on the mechanism of ligand transfer, there may be structural requirements for membrane interaction such as observed in sterol carrier protein-2 (*1*), CRBP I (*2*), iFABP (*3*, *4*), and phosphatidylcholine transfer protein (*5*). Many structural themes have evolved to fulfill such requirements for different hydrophobic ligands, and there are several examples of structural conservation among lipid binding protein families such as retinoid binding proteins (*6*, *7*) and FABPs (*3*).

Another family of lipid binding proteins consists of (among others) the cellular retinaldehyde binding protein (CRALBP)[1] (*8*, *9*), the yeast phosphatidylinositol transfer protein Sec14p (*10*), SPF (also referred to as TAP) that has been associated with both squalene (*11*) and tocopherol (*12*) metabolism, and the α-tocopherol transfer protein, α-TTP (*13*). These proteins contain the CRAL-TRIO domain (Pfam entry: PF00650) (*14*), a substructural scaffold based on the sequences of the CRALBP and the triple function domain of the Trio protein (*15*, *16*). The homologous CRAL-TRIO domain within these lipid binding proteins is suggested to encompass the ligand binding pocket based on the three-dimensional structures of Sec14p (*17*) and SPF (*18*).

Tocopherol binding proteins must potentially contend with a number of closely related compounds. Vitamin E is not a single compound, but rather consists of a family of hydrophobic tocols having differentially methylated chromanol headgroups giving rise to α-, *β*-, *γ*-, and *δ*-tocopherols. Unsaturation in the phytyl tail differentiates the tocotrienols from the tocopherols. It has long been recognized that mammals possess a mechanism that results in the selective physiological retention of *RRR*-α-tocopherol regardless of dietary intake of the different forms (*19*−*22*). Indeed, the naturally occurring *RRR*-α-tocopherol is the most efficient vitamer in reversing the pathologies associated with vitamin E deficiency (*22*−*25*).

α-TTP is a mammalian protein that was purified from liver based on its tocopherol binding activity (*26*−*30*). Examination of the ability of different tocopherol derivatives to compete the in vitro, TTP-catalyzed transfer of α-tocopherol between liposomes and mitochondria indicated a strong correlation between the affinity of a tocol to α-TTP and its activity in bioassays (*31*). Thus, it is generally postulated that TTP is responsible for the observed physiological selectivity for the *RRR*-α isomer of vitamin E (*22*). In support of this notion, humans with heritable mutations in the TTP

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[1] Abbreviations: AVED, ataxia with vitamin-E deficiency; CRALBP, cellular retinaldehyde binding protein; PI, phosphatidylinositol; PITP, phosphatidylinositol transfer protein; PC, phosphatidylcholine; SPF, supernatant protein factor; α-TTP, α-tocopherol transfer protein; TAP, tocopherol associated protein.

10.1021/bi034086v CCC: $25.00 © 2003 American Chemical Society
Published on Web 05/09/2003

gene exhibit altered ability to discriminate between the different tocols (*32*−*34*). However, no work has been reported to date where TTP's ligand selectivity has been directly measured by quantifying its dissociation constants for the different tocols.

SPF was recently identified based on its ability to bind tritiated α-tocopherol (*12*, *35*). Interestingly, the very same protein was identified and purified more than 25 years ago (*36*, *37*) based on its ability to stimulate microsomal squalene epoxidase, and it was later cloned (*11*). Since no direct measurements of the binding between SPF and different ligands have been reported to date, the nature of the "true" ligand of this protein is, at present, an enigma.

Ligand transfer between membranes can be thought of as two steps: association between the binding protein and the ligand, and the subsequent transfer of the ligand to the "acceptor" membrane. It should also be noted that the different properties of the tocopherols (hydrophobicity, steric bulk, and chirality) may affect the thermodynamics of insertion into membranes. Thus, dissecting the transfer reaction into two separate processes (binding vs transfer) is of great value for determining the specificity of α-TTP and SPF for individual ligands, and to assess their physiological relevance by comparison with related proteins such as Sec14p or CRALBP.

We wished to characterize the ligand specificity of α-TTP and SPF for natural forms of vitamin E to help support the postulated roles of these proteins in tocopherol metabolism (*22*, *38*−*40*). We chose to also include in our comparison two other members of the CRAL-TRIO family, Sec14p and CRALBP, since they are likely to share some structure/function features. Thus, the ligand binding profiles across the family would provide a context for assessing the promiscuity of ligand binding among structurally similar proteins. We have utilized a Lipidex-based binding assay to directly measure the ligand selectivity of members of the CRAL-TRIO family of lipid binding proteins. To aid in this comparison, we also developed a structural model of α-TTP based on the three-dimensional structure of Sec14p.

MATERIALS AND METHODS

Protein NCBI Accession Numbers. Human α-TTP: P49638, Sec14p: CAA33511, SPF: NP-036561; O76054, CRALBP: L34219.

Materials. Lipidex (hydroxyalkoxypropyl dextran, substituted with 10 wt % with alkyl chains of length C_{15}−C_{18}) was purchased from Canberra Packard or Sigma Chemical Co. Scintiverse scintillation cocktail was obtained from Fisher Scientific. All other reagents were obtained from Sigma Co. (Mississaugua, ON).

Recombinant Protein Expression and Purification. John Saari (Department of Ophthalmology, University of Washington) generously contributed purified recombinant human cellular retinaldehyde binding protein. The *S. cerevisiae* Sec14 gene was kindly provided by Marcus Ebneth, SunGene, Germany, in pQE-30 and by Vytas Bankaitis (University of North Carolina, Chapel Hill, NC). The pET-28 TTP construct was described previously (*41*). The SPF gene in pET-30a was the generous gift of A. Stocker (University of Bern, Switzerland). To verify that the poor tocopherol binding activity exhibited by SPF is not an artifact of the C-terminal histidine tag, three additional SPF constructs were generated with different amino-terminal histidine tags that leave 1, 4, or 14 amino acids after thrombin cleavage and purification. The binding of [^{3}H]-α-tocopherol to the purified SPF proteins obtained from all four constructs were determined and compared. The C-terminal His-tagged fusion protein from pET-30a demonstrated the most competent binding profile and highest affinity for α-tocopherol. This fusion protein was selected for competition experiments with additional ligands, which were repeated using protein isolated from two separate purified preparations. Protein purification methods were described in a previous publication (*41*). All constructs were sequenced prior to expression and purification. All protein concentrations were quantified with the Bradford protein assay (Bioshops, Burlington, ON).

Binding Assays. Homologous and heterologous competition experiments with [^{3}H]-*RRR*-α-tocopherol (51.6 Ci/mmol) were used to determine ligand dissociation constants by adaptation of the Lipidex assay previously reported by Timmers et al (*41*, *42*). Competitive experiments were chosen since they allowed for a wider concentration range of ligand than saturation experiments (*43*). Briefly, the sample containing [^{3}H]-*RRR*-α-tocopherol and protein was applied onto a Lipidex column (∼200 mg), allowed to drain, and then washed with 25 mM Tris-HCl, pH 7.4, 0.1 M KCl, 100 μM Triton-X100, 1 mM EDTA. The hydrophobic matrix retains the free tocopherol and allows the protein bound tocopherol to be collected. After washing of the sample, the matrix-bound tocopherol was eluted with 100% methanol into a fresh vial. Samples were equalized for solvent quenching (adding MeOH to the wash buffer and buffer to the MeOH fraction) and counted in a Beckman LS6500 scintillation counter. The recovery of radioactivity was always >95%.

Assays were conducted with 1.8−2.5 nM [^{3}H]-α-tocopherol and 0.1−0.2 μg of protein in a reaction volume of 320 μL, giving a final protein concentration from ∼9−17 nM. Only 250 μL was applied to the Lipidex column. Unlabeled competitors were added to achieve 8−10 points spanning the concentration range of roughly 1.8 nM to 50 μM of ligand. All competitors except for phospholipids were added from 100% ethanol stocks to maintain a concentration of ethanol in the final assay below 1%. Bovine liver phosphatidylinositol and phosphatidylcholine (Avanti Polar Lipids, Alabaster, AL) were dried overnight under vacuum, and the film was resuspended in incubation buffer with gentle vortexing. The suspension was sonicated to achieve a clear solution of unilamellar vesicles. All assay components were mixed, and samples were incubated at 37 °C for 2−2.5 h and placed on ice for 10 min before application to the Lipidex columns at 4 °C. All assays were performed in triplicate in at least three separate experiments, two in the case of CRALBP. Competition with α-tocopherol was done with each new ligand as a positive control. Assays were also performed with bovine serum albumin and lysozyme as negative controls. Nonspecific binding to the matrix was measured using the identical procedure in the absence of protein. This amount varies depending on the concentration of added cold tocopherol but ranged from 2 to 10% of apparent bound counts on any given experiment.

Binding Assays with SPF. Assays were performed with [^{3}H]-α-tocopherol, [^{3}H]-α-tocopheryl quinone, and [^{3}H]-squalene as above with the exception that 0.4 μg of protein

was used. Binding assays of SPF with α-tocopherol were also performed in the presence of 5 mM $CaCl_2$, 5 mM $MgCl_2$, 10 mM GTP, 10 mM GDP, $CaCl_2$ + GDP, $CaCl_2$ + GTP, $MgCl_2$ + GDP, and $MgCl_2$ + GTP.

Data Analysis. Normalized cpm data, corrected for solvent quenching, were fitted to either homologous (for α-tocopherol) or heterologous (for all other ligands) competition models using the treatment described by Cheng and Prusoff (*44*) for a one binding site binding model. Goodness-of-fit was calculated by the software (Prism 4.0, GraphPad, Inc.) and the correlation coefficients were always >0.92. All results are presented as the mean of triplicate measurements ± standard error.

Tritiated α-Tocopherol. [^{3}H]-6-*O*-*tert*-butyl dimethylsilyl-α-tocopherol (TBDMS-α-tocopherol) was prepared by Amersham-Pharmacia Life Sciences (Darbyshire, UK) by reduction of the 6-*O*-*tert*-butyl dimethylsilyl α-tocopherol chromene with $^3H_2$ and Pd/C. Prior to use, [^{3}H]-TBDMS-α-tocopherol was deprotected in tetrahydrofuran using *tert*-butylammonium fluoride, subsequently acidified with a few microliters of 1 N HCl, evaporated under a stream of nitrogen, and purified on a small silica column (hexane:ethyl acetate 15:1). Purified fractions were evaporated and resuspended in ethanol. Purity of the [^{3}H]-α-tocopherol was verified by TLC before use. Using unlabeled α-tocopherol for identification, the TLC plate was sectioned after development and each segment counted. To confirm that the [^{3}H]-α-tocopherol survived throughout the assay, mock samples were extracted with ether under acidic conditions in the presence of KCl and butylated hydroxytoluene both prior to and after incubations. The recovered tocopherol was a minimum of 95% pure.

Additional Tritiated Ligands. [^{3}H]-α-tocopheryl quinone was prepared by oxidation of approximately 800 nmol α-tocopherol in 50% ether, 45% MeOH, 5% water with 2 μmol $FeCl_3$ for 1.5 h at room temperature. The quinone was purified from the reaction mixture by silica chromatography (5:1 hexane/EtOAc) and selected fractions were evaporated and redissolved in ethanol. Purity of the quinone was ≥ 90%. Tritiated squalene (30 Ci/mmol) was obtained from American Radiolabeled Chemicals (St. Louis, MO). Binding assays were performed as with [^{3}H]-α-tocopherol by replacing the radioligand with either [^{3}H]-squalene or [^{3}H]-α-tocopheryl quinone (51.6 Ci/mmol).

Construction of a Model of α-TTP. All calculations were performed on a Silicon Graphics R4400 Indigo2 workstation. The modeling software used was QUANTA 4.1 (*45*) with the CHARMm 23.1molecular mechanics force field (*46*).

The three-dimensional structure of the phosphatidylinositol-transfer protein Sec14p from *Saccharomyces cerevisiae* was used as the template protein for the comparative model (RCSB−PDB code 1AUA) (*17*, *47*). A local pairwise alignment of α-TTP and Sec14p was done, using the SIM (*48*) alignment algorithm. The SIM local alignment employed the BLOSUM62 matrix, with gap open and extension penalties of 12 and 4, respectively. The best local sequence alignments were for residues 34−48, 53−85, and 176−268 of Sec14p, corresponding to residues 25−39, 45−79 and 157−249, respectively, in α-TTP. A subsequent global alignment was performed using QUANTA 4.1, with the PAM250 matrix, a gap open penalty of 5, and a gap extension penalty of 5. The global alignment was entirely consistent with the local alignment, in that the three regions of best local alignment were maintained.

Residues in α-TTP were placed in three-dimensional space by assigning the coordinates of the aligned Sec14 residues. Nonidentical side chain residues were positioned in an extended conformation. Only four residues in α-TTP (Pro 41, Ala 176, Pro 46, and Leu 47) had undefined coordinates, due to gap insertions in the Sec14p alignment. Coordinates for the atoms in these residues were found either by searching a fragment database or by simple coordinate assignment. Energy minimization of isolated peptide fragments comprised of the newly generated residues plus two or three amino acids on either side, relieved internal coordinate strain and unfavorable steric interactions.

The initial coordinate assignment of α-TTP, based on the global alignment with Sec14p, left 10 physical gaps interspersed throughout the three-dimensional structure, where adjacent residues were separated by spacings from one to three amino acid residues in length. These physical gaps were closed by rounds of energy minimization, to arrive at a preliminary α-TTP model having contiguous backbone coordinates.

RESULTS

Sequence Alignment and Modeling of α-TTP. A BLAST search using the TTP sequence identified several members of the CRAL-TRIO family, including Sec14p, SPF, and the human cellular retinaldehyde binding protein (CRALBP), the MEG2 tyrosine phosphatase, the squid retinal binding protein, as well as some other sequences whose identity and function are still unknown. The sequence elements common to members of this family are derived from the sequences of CRALBP and the signaling domain of the multidomain TRIO protein (*14*), and were therefore assigned the motif name CRAL-TRIO (Pfam: PF00650). The CRAL-TRIO family shares an approximately 185-amino acid sequence that includes the SEC14 carboxy-terminal domain (Figure 1). Generally, CRAL-TRIO motifs are believed to function in lipid binding. Specifically, the CRAL/TRIO domains of Sec14p, SPF, CRALBP, and α-TTP are likely to constitute the region of ligand binding based on the three-dimensional structures of Sec14p and the recently described structure of SPF (*18*). The relevance of this domain for ligand transfer activity is further supported by the existence of homologous mutations within this region in CRALBP (*49*), Sec14p (*47*), and α-TTP (*50*) that are known to sacrifice transfer of their appropriate ligands. Figure 2 shows the ribbon diagrams of the calculated model of α-TTP, the three-dimensional structure of Sec14p obtained by X-ray diffraction, and a calculated model of SPF using the SwissModel tool (*51*). The residues of α-TTP that have been identified in individuals having ataxia with vitamin E deficiency (AVED) are highlighted. The missense mutations of K66,239A in Sec14p are known to be required for transfer of PI (*52*). Inspection of the structural model of Sec14p shows that K239 is homologous to one of the AVED mutations, R221, in α-TTP, as well as to K216 in SPF. R65 of Sec14p may also be important given the conservation of this residue (Figure 1) in TTP (R59), CRALBP (R103), and SPF (R45).

The α-tocopherol molecule was oriented in the active site of α-TTP by overlapping the chromanol headgroup with one of the β-octylglucoside ligands (BOG1) in the Sec14p

FIGURE 1: ClustalW alignment of human α-TTP, hCRALBP, *S. cerevisiae* Sec14p, and SPF depicting the region of greatest homology between the four proteins within the CRAL-TRIO and SEC14 domains. Similar residues are shaded gray and conserved residues are shown in black boxes. Crosses (†) denote the residues in α-TTP that are mutated in AVED.

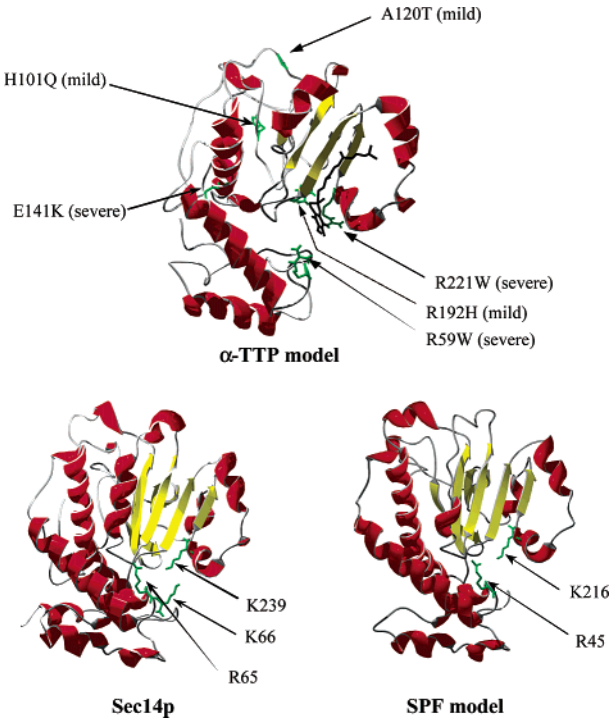

FIGURE 2: Comparison of the structural models of α-TTP and SPF with the Sec14p 3D structure. The α-TTP model was generated using the Sec14p crystal structure as a template (pdb code: 1AUA). SPF was modeled using the Swiss-Model tool and includes residues 1−275. Location and conformation of *RRR*-α-tocopherol (black) was obtained by preliminary docking calculations (manuscript in preparation). The chromanol group was superimposed on the coordinates of the bound *β*-octyl glucoside (BOG-1). Point mutations that result in ataxia with vitamin E deficiency (AVED) are shown for α-TTP, and those that disrupt transfer of phosphatidylinositol in Sec14p. Two analogous mutations are shown for SPF.

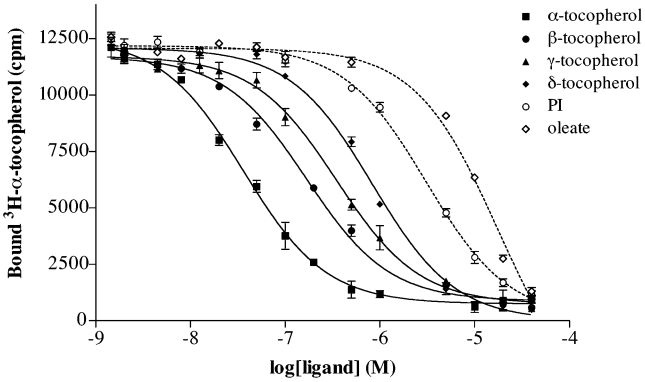

FIGURE 3: Representative competitive binding curves for human recombinant α-TTP with tocopherols (—) and the nonspecific ligands phosphatidylinositol and oleic acid (- - -). α-TTP (0.1 *μ*g) was incubated with 1.8−2.5 nM [^{3}H]-α-tocopherol (51.6 Ci/mmol) and the indicated concentrations of unlabeled competitors for 2 h at 37 °C. Binding assays were performed in triplicate in at least three independent experiments. Error bars indicate the standard error of the mean.

structure. BOG1 was chosen because it provided more room for the phytyl tail, which extends along the length of the binding site. It has also been proposed that PI adopts a similar orientation in Sec14p (*17*). The illustrated conformation of α-tocopherol within the putative binding site of α- TTP was the best found during preliminary Monte Carlo/molecular dynamics docking calculations. Of 1400 randomly docked, relaxed, and energy-minimized conformations, this conformation and position of α-tocopherol has the lowest interaction energy with the protein, a low internal strain energy, in addition to having a high fraction of its available surface area in contact with the protein.

Homologous Competition with α*-Tocopherol for Human* α*-TTP*. The homologous competition experiments for α-tocopherol with human α-TTP yielded a K_d of 25.0 ± 2.8 nM as illustrated in Figure 3. The dissociation constant was derived from 10 independent experiments, each performed in triplicate. This value is comparable to that of recombinant CRALBP for its preferred ligand 11-*cis*-retinal (K_d 21 nM) (*53*), and for the FABPs affinities for some fatty acids (*54*−*57*).

Inhibition Constants of Other Tocopherols for α*-TTP*: *Role of Chroman Methylation*. The binding constants for α-TTP with other tocopherols are illustrated in Table 1. An important

Table 1: Comparison of Dissociation Constants of Recombinant Human α-TTP, *S. cerevisiae* Sec14p, SPF, and CRALBP for Various Hydrophobic Ligands

ligand	dissociation constant (nM)[a]				relative affinity of TTP for α-tocopherol[b]	rat-resorption gestation assays [c]
	α-TTP	Sec14p	SPF	CRALBP		
α-tocopherol	25.0 ± 2.8	373 ± 89	615 ±15	528 ± 9	100	80
β-tocopherol	124 ± 4.7	3914 ± 286	393 ± 32	nd[d]	38.1 ± 9.3	45
γ-tocopherol	266 ± 9	3990 ± 420	268 ± 13	nd	8.9 ± 0.6	13
δ-tocopherol	586 ± 75	3908 ± 900	731 ± 82	nd	1.6 ± 0.3	<0.4
SRR-α-tocopherol	545 ± 62	nd	nd	nd	10.5±0.4	59 (d/l)
Trolox	1004 ± 126	ic	nd	nd	9.1 ± 1.2	
α-tocopheryl acetate	1639 ± 89	5559 ± 900	nd	nd	1.7 ± 0.1	136
α-tocopheryl succinate	526 ± 54	nd	nd	nd		
6-*O*-carboxymethyl-α-tocopherol	879 ± 65	nd	nd	nd		
α-tocotrienol	214 ± 13	4726 ± 884	nd	nd	12.4 ± 2.3	13
α-tocopheryl quinone	814 ± 86	5701 ± 395	441 ± 4	nd		
cholesterol	ic	ic	nd	nd		
oleic acid	7200 ± 1030	ic	ic	nd		
squalene	ic	ic	879 ± 123	nd		
n-*β*-octyl glucopyranoside	ic	ic	nd	nd		
phosphatidylinositol	1415 ± 106	381 ± 43	216±31	nd		
phosphatidylcholine	nd	6123 ± 234	1183±178	nd		
9-*cis*-retinal	786 ± 67	nd	nd	67.5 ± 1.9		
trans-retinol	ic	ic	nd	nd		
retinoic acid	ic	ic	nd	nd		

[a] All data are expressed as the average ± SEM. [b] Ref *31*. [c] Ref *58*. [d] nd = not determined; ic = incomplete competition; competition was not achieved at 20 *μ*M [ligand].

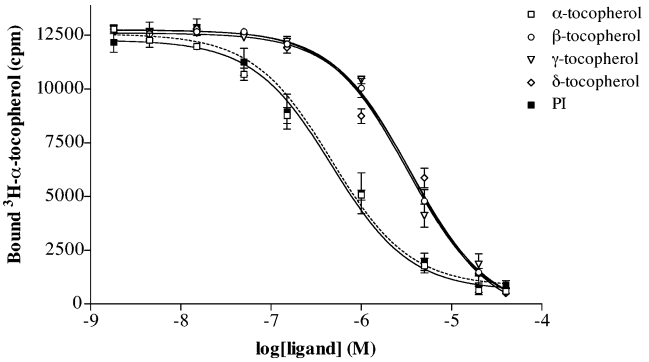

FIGURE 4: Representative competition curves for recombinant *S. cerevisiae* Sec14p with tocopherols (—) and phosphatidylinositol (- - -). Sec14p (0.4 *μ*g) was incubated with 1.8−2.5 nM [^{3}H]-α-tocopherol (51.6 Ci/mmol) with the indicated concentrations of unlabeled competitors for 2 h at 37°C. Binding assays were performed in triplicate in at least two independent experiments. Error bars indicate the standard error of the mean.

observation from these data is that the extent and position of methylation of the chroman ring system influences binding. Specifically, α-TTP demonstrates a significant preference for α-tocopherol (25 nM) followed by *β*- (124 ± 4.7 nM), *γ*- (266 ± 9 nM), and finally *δ*-tocopherol (586 ± 75 nM). This ranking of binding affinities correlates well with the reported ability of these molecules to inhibit the in vitro α-TTP-catalyzed α-tocopherol transfer between membranes (*31*), the biological activities as determined from rat gestation-resorption assays (*58*) and the biokinetics of α- and *γ*-tocopherol (*59*, *60*). *β*-Tocopherol, which is methylated at the C5-position, had a 2-fold higher affinity for α-TTP than *γ*-tocopherol that lacks this substituent. The presence of only a single methyl group in *δ*-tocopherol resulted in a 250-fold decrease in affinity to α-TTP. A greater diminution in the binding affinity is observed for the 2S stereoisomer (K_d 545 ± 62 nM) than for loss of the single C5 methyl group in *γ*-tocopherol. The importance of the free phenol for ligand binding is exemplified by the poor recognition of α-tocopheryl acetate, α-tocopheryl succinate, 6-*O*-carboxymethyl α-tocopherol, and α-tocopheryl quinone, although lower affinity of these ligands could also result from steric effects and gross structural changes.

Variations within the phytyl chain show disparate effects. As illustrated by the binding of Trolox, a water-soluble, side chain-truncated chromanol, removal of the phytyl chain results in some 40-fold decrease in affinity relative to α-tocopherol. The unsaturated side chain of α-tocotrienol reduced its affinity to α-TTP 8-fold versus α-tocopherol, but α-tocotrienol remained a better ligand than α-tocopheryl acetate, *γ*- or *δ*-tocopherol. It is worth noting that the efficacies of Trolox and α-tocotrienol as inhibitors of α-TTP-catalyzed inter-membrane transfer are about the same (*31*), despite their significant differences in binding affinity for α-TTP (Table 1).

Several other lipophilic ligands were evaluated to assess the ligand promiscuity of α-TTP. As shown in Table 1, most of these ligands failed to compete with [^{3}H]-tocopherol even at high concentrations. α-TTP demonstrated a K_d of 1.41 ± 0.21 *μ*M for phosphatidylinositol, similar to α-tocopheryl acetate, yet almost 50-fold weaker than the affinity observed with α-tocopherol. Similarly, α-TTP was capable of binding 9-*cis*-retinal with a K_d of 586 ± 69 nM, suggesting that the homology within the ligand binding pocket across these members of the CRAL-TRIO family is sufficient for substantial overlap in ligand specificity under the experimental conditions employed here.

Ligand Selectivity With Sec14p. Evaluation of the binding of various ligands to Sec14p indicated a surprisingly high affinity for α-tocopherol (373 ± 89 nM) relative to that for phosphoinositide (381 ± 43 nM). Sec14p binds α-tocopherol approximately 10-fold better than the other tocopherols tested, whereas *β*-, *γ*-, and *δ*-tocopherol are equally poor ligands (K_d > 1.5 *μ*M). The binding affinities of the different tocopherols for Sec14p do not reflect those for α-TTP in order or in magnitude. Yet, like α-TTP, Sec14p demonstrates a preference for ligands with a free phenol; α-tocopheryl

acetate and α-tocopheryl quinone exhibit the lowest affinity for Sec14p (>5 μM). Binding assays with phosphatidylinositol (PI) and phosphatidylcholine (PC) confirmed that recombinant Sec14p bound its preferred ligand PI with a K_d of 381 ± 43 nM in contrast to a K_d of 6123 ± 234 nM for PC. The ~16-fold higher affinity for PI correlates well with its 19-fold faster inter-membrane transfer by Sec14p (*61*).

Ligand Binding by SPF. Recombinant SPF demonstrated a dissociation constant of 615 ± 68 nM for α-tocopherol, similar to a previous report of 460 nM using surface plasmon resonance with immobilized tocopherol (*12*). SPF has also been reported to transfer squalene, but stoichiometric binding could not be demonstrated (*11*). In our hands, SPF had a K_d for squalene of 879 ± 123 nM, similar to that for α-tocopherol.

Previous reports suggest that SPF contains a GTP binding motif (*12*, *35*), offering the possibility that small molecules may modulate binding of α-tocopherol to the protein. We have investigated this notion by repeating the α-tocopherol binding assays in the presence of 2 mM Ca^{2+} or Mg^{2+}, 10 mM GTP or GDP, and combinations thereof. None of these treatments had a significant effect on the affinity of SPF for α-tocopherol (data not shown).

Given the prolonged incubations of protein and [^{3}H]-α-tocopherol reported in a previous study (*12*) and our experience with the chemical instability of tocopherol in aqueous solutions, we investigated the possibility that SPF may bind oxidized forms of tocopherol more efficiently than the reduced form. SPF, Sec14p, and α-TTP were incubated at room temperature for 24 h or for 2 h at room temperature, and ligand binding was assayed as described above. SPF bound about twice as much radioactivity at 24 h as at 2 h, whereas α-TTP bound half as much and Sec14p bound the same amount of radioactivity in these two incubations. The diminished binding to α-TTP after 24 h correlated well with the extent of tocopherol oxidation as assayed by TLC (data not shown). To further establish SPF's ability to bind oxidized tocopherol, pure [^{3}H]-α-tocopheryl quinone was prepared and used as ligand in homologous binding assays with cold α-tocopheryl quinone. SPF displayed an approximately 30% greater affinity for α-tocopheryl quinone (K_d = 441 ± 4 nM) than for α-tocopherol (615 ± 15 nM).

As a further measure of binding specificity, heterologous competition with other tocopherols showed γ-tocopherol (K_d 268 ± 13 nM) to be the most competent ligand in the vitamin E family. SPF displayed a unique profile of tocopherol binding compared to Sec14p or TTP, with the order of binding affinities proceeding as γ (268 ± 13 nM) > β (393 ± 32 nM) > α (615 ± 15 nM) ~ δ (731 ± 82 nM). This observed preference of SPF for γ-tocopherol agrees with the previous binding investigations of SPF (*12*). Homologous competition with [^{3}H]-squalene yielded a K_d of 879 ± 123 nM substantiating that SPF can bind both squalene and α-tocopherol with similar affinities. However, PI was the most competent ligand studied with a binding affinity (K_d 216 ± 31 nM) comparable to γ-tocopherol. Oleic acid failed to compete with α-tocopherol for binding to SPF.

DISCUSSION

The dissociation constants of recombinant human α-TTP for different tocopherols correlate well with those derived previously from inhibition of α-TTP-mediated intermembrane transfer assays (*31*) and from their respective efficacies in rat fetal resorption experiments (*58*). Thus, α-TTP's ligand specificity supports the notion that α-TTP is an important discriminating physiological factor for the specific retention of dietary α-tocopherol (*22*) (Table 1). This is demonstrated particularly well in the case of α- and γ-tocopherol where the ratio $K_d\gamma/K_d\alpha = 10.6$, and the ratio of α- to γ-tocopherol concentrations in human plasma was reported to be in the range of 7:1 to 18:1 (*62*, *63*). The ligand selectivity also highlights the structural features important for ligand recognition, namely, an appropriate methylation pattern on the chromanol ring. The absence of a single methyl group at C7 in β-tocopherol and at C5 in γ-tocopherol results in a loss of free energy of binding of about 1.0 and 1.5 kcal/mol, respectively. δ-Tocopherol, which is missing two methyl groups relative to α-tocopherol, has a free energy of binding about 2 kcal/mol less than α-tocopherol. This is somewhat remarkable given that the free energy of binding for α-tocopherol to α-TTP under our conditions is about 11 kcal/mol. While the phytyl tail is required for higher affinity binding (i.e., Trolox vs α-tocopherol), α-TTP tolerates some structural variation within this element. For instance, the conformational differences introduced by the unsaturated side chain of α-tocotrienol do not lead to a drastic loss of binding affinity. The effect of α-tocopherol on lipid order and acyl chain flexibility in phospholipid bilayers is beginning to be appreciated (*64*, *65*), and it may be that the hydrophobicity, steric bulk, rigidity, and/or chirality of different tocopherols effects the ease of transfer to acceptor membranes. Thus, it remains possible that binding studies do not fully describe the intermembrane transfer reaction. However, the observed correlation between our measured affinities of α-TTP for various ligands with their reported efficacy in bioassays suggest that protein−ligand recognition alone is likely to be a significant component of the physiological discriminatory mechanism.

We initially used Sec14p as a control for our binding measurements with α-TTP. Surprisingly, Sec14p exhibits measurable and significant affinity for α-tocopherol, presumably because of the structural similarity between the binding pockets of Sec14p and α-TTP. Hence, we were prompted to explore the binding behavior of additional homologous proteins. Of the ligands and proteins tested, Sec14p bound α-tocopherol most competently with a K_d ~ 15-fold lower than that of α-TTP. Comparatively, α-TTP exhibited a 4-fold lower affinity for PI than did Sec14p. Measurements of the affinities of phospholipids to α-TTP and Sec14p by the Lipidex assay may be further complicated by the presence of an additional phase, the phospholipid vesicles. We were concerned that the presence of vesicles or aggregates of phospholipid would interfere with our binding assays. Thus, we performed protein-free control incubations for each point in assays in the presence of phospholipid. The tritiated tocopherol that flowed through the Lipidex under these conditions was subtracted from that observed in the presence of protein. Because of this correction, the true dissociation constants for PI and PC may be lower than shown in Table 1.

Despite such possible underestimation of the absolute affinities, Sec14p displayed a ~16-fold higher affinity for PI relative to that for PC. This is consistent with the 19-fold higher intermembrane transfer rate of PI over PC observed with Sec14p in an early report (*61*). Finally, Sec14p

demonstrated a greater affinity for tocopherol than for other lipophilic ligands such as oleic acid, squalene or *trans*-retinol. This implies that the ligand binding site of Sec14p manifests a true selectivity for α-tocopherol, rather than simply being capable of binding linear hydrophobic molecules.

The promiscuity of members in the CRAL-TRIO family in binding tocopherol is further supported by our binding measurements with CRALBP which bound α-tocopherol with a K_d of 528 ± 9 nM as compared to a K_d of 68 ± 2 nM for 9-*cis*-retinal. The dissociation constant of bovine CRALBP for 9-*cis*-retinal was reported to be 53 nM, ~10-fold more competent a ligand than α-tocopherol (*53*). The binding of both PI and PC with a variety of acyl chains by Sec14p requires greater flexibility within the binding cavity surrounding both the polar headgroups of the ligand and the hydrophobic tails. The promiscuity of the PITP family has been previously noted, as they appear to always be purified with a bound ligand. Recombinant proteins are often found complexed with bacterial lipids or detergents upon isolation from *Escherichia coli*, as seen in the case of Sec14p and SPF (*47*, *66*). Similarly, α-TTP, while binding all members of the tocopherol family, has the specificity required to discriminate for α-tocopherol.

The metabolism of α-tocopherol by microorganisms has not been extensively examined, although yeast possess the vitamin as a membrane component (*67*). The phosphatidylinositol transfer protein of *S. cerevisiae* is not structurally related to mammalian phospholipid tranfer proteins, PITPα and PITP*β*, although both exhibit higher affinity toward PI that toward PC (*68*). It has been shown that Sec14p can complement PITPα depleted mammalian cells, and that PITP can rescue Sec14 yeast null mutants (*47*). There are four Sec-like proteins of unknown function in humans that contain the CRAL-TRIO motif. One of these was recently cloned and the gene was postulated contribute to retinal degeneration (*69*). No functional investigations of any of these proteins have been reported.

SPF demonstrated weak affinity for α-tocopherol compared to Sec14p and CRALBP. In contrast to Sec14p or α-TTP, SPF did not exhibit significant selectivity toward α-tocopherol, and it is therefore unlikely that this protein mediates the known biological activities of this vitamin. It appears that protein sequence homology does not necessarily manifest in similar ligand specificity; however, the structural similarity of the four members of the CRAL-TRIO family is sufficient to allow recognition of common ligands. Promiscuity in ligand binding is not a novel occurrence among lipid-binding proteins. The lipocalin family (*70*, *71*) is an excellent example of proteins with similar tertiary structures despite low sequence similarity. Although cross-family ligand binding is not usually investigated for proteins with diverse functions, the present work offers an example of the complexity of deriving functional properties from sequence comparisons alone or from the measurement of binding of a single ligand. An additional example is the structure−function relationship of five yeast Sec-like proteins isolated based on homology to Sec14p (*72*). Interestingly, the protein exhibiting the lowest sequence similarity to Sec14p (21%), Sfh5p, demonstrated the highest PI transfer activity in contrast to the protein of the highest sequence identity (64%), Sfhp1, which possessed no observable PI binding.

The addition of nucleotides (GTP, GDP) or cations (Mg^{2+}, Ca^{2+}) to the binding assays as possible modulators for SPF did not alter its affinity for α-tocopherol. Similar supplementation with guanine nucleotides also failed to activate the ability of SPF to stimulate squalene monooxygenase (*73*). However, inclusion of ATP and cytosolic extracts or exogenous kinases did lead to an increase in the latter activity (*73*), suggesting a possible involvement of posttranslation modifications (such as phosphorylation) in modulating the biochemical properties of SPF. It remains to be determined whether tocopherol/squalene binding by SPF is also modulated by such factors. The binding of other ligands indicated that although SPF displayed only modest preference for tocopherols, *γ*- rather than α-tocopherol was the most competent ligand, while equal recognition of PI and *γ*-tocopherol was observed. We have determined that SPF exhibits a measurable (albeit weak) affinity for squalene, in line with a previous report where squalene transfer activity of SPF was described (*11*).

The mechanism underlying intracellular trafficking of α-tocopherol remains largely unknown. It has been suggested that SPF may contain a GTP-binding consensus motif (*12*, *35*), and the three-dimensional structure (*18*) illustrates a unique C-terminus extension of unknown function, implying that there may be more to SPF's function and regulation than "simply" binding tocopherol or squalene. Overall, our data demonstrate a lack of clear ligand selectivity and are suggestive of additional regulatory mechanisms or that SPF's "true" physiological ligand still remains to be identified. Clearly, more experimental work is needed before conclusive statements about SPF's function(s) are made.

Comparison between missense mutations in the α-TTP gene, which cause ataxia with vitamin E deficiency (AVED) and point mutants of Sec14p that interfere with PI transfer, lends support to similar mechanisms of ligand transfer for both proteins. Of the six point mutants contributing to the AVED pathology, two responsible for early onset disease correspond with Sec14p mutants of diminished function: R59W and R221W in α-TTP (*50*) and the corresponding K66A and K239A in Sec14p (*52*). Mutations within the CRALBP ligand-binding domain possibly contribute to pathological conditions. For example, R234W substitution in CRALBP yields a nonfunctional protein that is associated with retinitis pigmentosa (*49*, *74*, *75*). Similar residues occur in Sec14p at R209 and in α-TTP at K190, although the importance of these residues has not been studied. How such shared mutational sensitivity explains the binding and transfer behaviors of α-TTP, CRALBP, Sec14p, and SPF await additional structural-function studies. This work supports the role of α-TTP in differentiating between dietary tocopherols, although question remains concerning how the membrane environment moderates tocopherol transfer and distribution. Further mechanistic studies of the membrane transfer activity of recombinant human and rat α-TTP is in progress using fluorescent tocopherol analogues.

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BI034086V