Molecular Determinants of Heritable Vitamin E Deficiency†

Samantha Morley,[#] Candace Panagabko,[‡] Diana Shineman,[#] Bernhard Mani,[§] Achim Stocker,[§] Jeffrey Atkinson,[‡] and Danny Manor*,[#]

Division of Nutritional Sciences, Cornell University, Ithaca, New York 14853 USA, Department of Chemistry and Centre for Biotechnology, Brock University, St. Catharines, Ontario, Canada, L2S 3A1, and Institute of Microbiology, Eidgenössische Technische Hochschule (ETH) Schmelzbergstrasse 7 CH-8092 Zürich, Switzerland

Received December 22, 2003; Revised Manuscript Received February 6, 2004

ABSTRACT: Tocopherol transfer protein (TTP) is a key regulator of vitamin E homeostasis. TTP is presumed to function by transporting the hydrophobic vitamin between cellular compartments, thus facilitating its secretion to the extracellular space. Indeed, recombinant TTP demonstrates marked ability to facilitate tocopherol transfer between lipid bilayers. We report the biochemical characterization of six missense mutations TTP[1] that are found in human AVED patients. We expressed the H101Q, A120T, R192H, R59W, E141K, and R221W TTP mutants in *Escherichia coli*, and purified the proteins to homogeneity. We then characterized TTP and its mutant counterparts with respect to their affinity for *RRR*-α-tocopherol and to their ability to catalyze tocopherol transfer between membranes. We observe the R59W, E141K, and R221W mutations, associated with the severe, early-onset version of AVED, are impaired in tocopherol binding and transfer activity. Surprisingly, despite the profound clinical effect of the R59W, E141K, and R221W mutations in vivo, their impact on TTP activity in vitro is quite benign (2−3-fold reduction in transfer kinetics). Furthermore, mutations associated with milder forms of the AVED disease, while causing pronounced perturbations in tocopherol homeostasis in vivo, are remarkably similar to the wild-type protein in the tocopherol transfer assays in vitro. Our data indicate that tocopherol transfer activity in vitro does not properly recapitulate the physiological functions of TTP. These findings suggest the possibility that the AVED syndrome may not arise from an inability of TTP to bind or to transfer alpha tocopherol, but rather from defects in other activities of the protein.

Vitamin E was originally discovered as a substance that prevented fetus resorption in diet-restricted rats (*2*). The term vitamin E is now known to represent a family of small plant lipids composed of a chromanol ring and a phytyl side-chain. Vitamin E molecules differ by the methylation pattern of their chromanol ring (α- vs *β*- vs *γ*- vs *δ*-), the degree of saturation of the phytyl tail (tocopherols vs tocotrienols), and the stereochemistry of three chiral centers in the molecule (*R* vs *S* stereoisomers). Members of the vitamin E family demonstrate potent radical trapping (i.e., antioxidant) activity both in vitro and in vivo (*3*, *4*), and it is generally accepted that this scavenging activity is at the basis for the physiological requirement for vitamin E. Studies in vitamin E-depleted rodents established the relative biological potency of tocopherols, and have shown that the naturally occurring TOH is some 10-fold more potent than other vitamin E forms (e.g., refs *5*−*7*). In addition, mammals selectively retain αTOH over other forms of vitamin E, regardless of relative dietary intake levels (*8*−*10*).

Because of its long in vivo retention time and its prevalence in virtually all diets, vitamin E deficiency is extremely rare in humans. AVED is a rare autosomal recessive disorder associated with low circulating αTOH levels, and neurodegenerative pathology that is presumed to result from increased oxidative stress (*11*, *12*). Additionally, some AVED patients are unable to discriminate between the different tocopherols (*13*). Mutations that cause AVED affect the expression and function of TTP, a 33-kDa member of the CRAL-TRIO family of lipid-binding proteins (*1*, *14*). Indeed, targeted disruption of the TTP gene in mice leads to diminished vitamin E levels in plasma and tissues (*15*−*17*), and to the clinical hallmarks of the AVED pathology (*18*). While TTP was originally believed to be expressed solely in liver, it is becoming apparent that its expression in other tissues is significant and physiologically important (*12*, *18*, *19*).

The "signature" activity of TTP, and the only known biochemical function of this protein, is facilitation of αTOH transfer between membranes (*20*−*22*). Accordingly, the role of TTP in vitamin E homeostasis is generally believed to involve transport of the hydrophobic ligand between different cellular compartments. The exact molecular mechanism by which TTP regulates vitamin E levels is unknown. Arita et

† S.M. was supported by Training Award T32-DK715827 from the National Institutes of Health.

* To whom correspondence should be addressed. Phone: (607) 255-6085; fax (607) 255-1033; e-mail: dm43@cornell.edu.

[#] Cornell University.

[‡] Brock University.

[§] Eidgenössische Technische Hochschule (ETH).

[1] Abbreviations: TTP, alpha tocopherol transfer protein; AVED, ataxia with vitamin E deficiency; TOH, RRR-α-tocopherol; GST, glutathione-*S*-transferase; IPTG, isopropyl-*β*-D-thiogalactopyranoside; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; TRIS, tris(hydroxymethyl)aminomethane; FPLC, fast performance liquid chromatography; CRALBP, cellular retinaldehyde binding protein; ABC-A1, ATP-binding cassette transporter A1.

10.1021/bi0363073 CCC: $27.50
Published on Web 03/20/2004

al. have shown that ectopic expression of TTP in cultured hepatocytes is accompanied by facilitated secretion of αTOH to the media (*23*). Since tocopherol secretion was resistant to brefeldin A treatment (i.e., independent from nascent VLDL secretion) the authors concluded that TTP functions in a novel, tocopherol-specific hepatic export pathway. The cellular components involved in this pathway, their mechanisms of action, and their modes of regulation are, at present, unknown. The exact mechanism by which TTP regulates this export pathway is similarly enigmatic.

Our long-term goal is to gain molecular-level understanding of the role of TTP in αTOH transport, its mechanism of action, and the AVED pathobiology. Toward this end, we present here the biochemical characterization of six naturally occurring substitution mutations in TTP that occur in some human AVED patients.

MATERIALS AND METHODS

Molecular Constructs. Cloning of the TTP gene into the pET28b vector was described earlier (*24*). To generate TTP mutants, site-directed mutagenesis of the wild-type gene was performed using the QuickChange kit and protocol (Stratagene). To enhance expression levels, and to minimize the number of non-native residues derived from the vector, the TTP genes were transferred to the pGEX-4T-3 vector (Pharmacia), using the Sal I (5′) and Not I (3′) restriction enzymes. In the pGEX fusion constructs, only six amino acid residues are attached to the purified protein after thrombin treatment and removal of the GST tag.

Protein Expression and Purification. pGEX constructs harboring the TTP variants were transformed into *E. coli* (JM109) cells, grown at 37 °C, and protein expression was induced with 0.5 mM IPTG at $OD_{600} = 0.6-0.8$. Bacterial cultures were harvested, washed once in buffer A (20 mM Tris, 1 mM EDTA, 1 mM DTT, 1 mM azide, 200 mM NaCl, pH 8.0), and stored at −20 °C until use.

Bacterial pellets were thawed and homogenized in 2× buffer A supplemented with 100 μM PMSF and 5 μg/mL of each aprotonin and leupeptin, sonicated, and centrifuged at 27000*g* for 30 min. The supernatant was incubated with glutathione agarose (10−20 mL slurry) for 4 h at 4 °C with constant mixing, and packed into a column. After washing of the sample with buffer B (buffer A supplemented with 0.1% Igepal and 1 mM DTT), bound protein was eluted with buffer A supplemented with 20 mM reduced glutathione. The eluted GST−TTP fusion protein was incubated with thrombin (30 U/mg fusion protein for the wild-type, H101Q, A120T, R192H, and R59K forms of TTP, 18 U/mg for the E141K, R59W, R221W, and R59A mutants) for 2 h at room temperature. The TTP was separated from the cleaved GST tag by two subsequent precipitations in 50% ammonium sulfate. The precipitated TTP was resuspended in buffer C (20 mM Tris, 150 mM NaCl, pH 8.0), dialyzed into storage buffer (20 mM Tris pH 8.0, 150 mM NaCl, 1 mM DTT, 50% (v/v) glycerol), and stored at −20 °C until use. Whenever necessary, high MW contaminants were removed by gel filtration on a Superdex 75 column (16−60, Pharmacia FPLC system). All preparations were >90% pure as judged by SDS−PAGE and Coomassie staining. Protein concentrations were determined with the Bio-Rad Protein assay (Bradford) kit, using bovine serum albumin as standard. Purified proteins underwent a significant (>50%) loss in tocopherol transfer activity if not transferred to −20 °C within 1 day of the last purification step. The TTP variants differed in their solubility in *E. coli*, and hence their respective yields. Specifically, yields of the GST−TTP fusion protein were as follows (mg of fusion protein per liter of *E. coli* culture): R192H > 15 mg/L; H101Q - 10 mg/L; A120T and wild-type - 5 mg/L; R221W - 3 mg/L; E141K and R59W - 1.5 mg/L.

Tocopherol Binding Assays. Affinities of the different proteins to tocopherol were determined by the Lipidex assay, using [^{3}H] *RRR*-α-tocopherol as described earlier (*1*, *24*).

Tocopherol Transfer Assays were performed as described by Hosomi et al. (*22*) with slight modifications. Briefly, sonicated unilamellar vesicles (liposomes) were prepared from egg-yolk phosphatidylcholine, dicetyl phosphate, and butylated hydroxytoluene (molar ratio 10:1:0.5), in the presence of [^{14}C]-alpha tocopherol (57 mCi/mmol, 2.4×10^6 cpm per mL liposomes) and [^{3}H]-triolein (as a nonexchangeable marker, 1 mCi/mmol, 1.3×10^6 cpm per mL liposomes) by sonication, and kept at 4 °C under argon atmosphere in the dark. Rat liver mitochondria were prepared according to (*25*), and stored at −80 °C. Before each use, aliquots of the mitochondria preparations were thawed and washed by centrifugation in buffer D (0.25 M sucrose, 1 mM EDTA, 70 mM Tris pH 7.5). In a typical transfer assay, liposomes (10 μmol of lipid) and mitochondria (75 μg of protein) were incubated for the indicated times in a total reaction volume of 1 mL buffer D at 25 °C. The reaction was initiated by addition of one microgram TTP, and terminated by a 30 min centrifugation at 17500*g*. Radioactivity (^{14}C and ^{3}H separately) was measured in the supernatant in a scintillation counter after addition of 4 mL scintillation cocktail (Ecoscint). More than 90% of the liposome radioactivity was accounted for in the supernatant. Tocopherol transfer activity was calculated as follows (*20*):

$$\text{transfer (\%)} = \left[\left(\frac{^3\text{H}/^{14}\text{C in presence of protein}}{^3\text{H}/^{14}\text{C in absence of protein}}\right) - 1\right]*100$$

Assays were performed in triplicates, from which averages and standard deviations were calculated. Data presented here were measured at least twice, each from at least two independent purified protein preparations (data for the E141K and R59W mutants are from one protein preparation, repeated three times in triplicates). Transfer kinetics were analyzed by fitting the time-dependent data into a single first-order process, from which the half-life ($t_{1/2}$) values were obtained.

Urea Unfolding Experiments. Protein samples (0.5 μM final concentration) were incubated in 20 mM Tris, 150 mM NaCl, pH 8.1 and the indicated concentration of urea for 19 h at room temperature. Protein fluorescence was measured in a Spex Fluorolog fluorimeter, using excitation wavelength of 294 nm. Emission spectra were collected in the 300−400 nm range (spectral resolution 3 nm).

RESULTS

Tocopherol Transfer Activity of Recombinant TTP. We recently described the cloning, overexpression, and purifica-

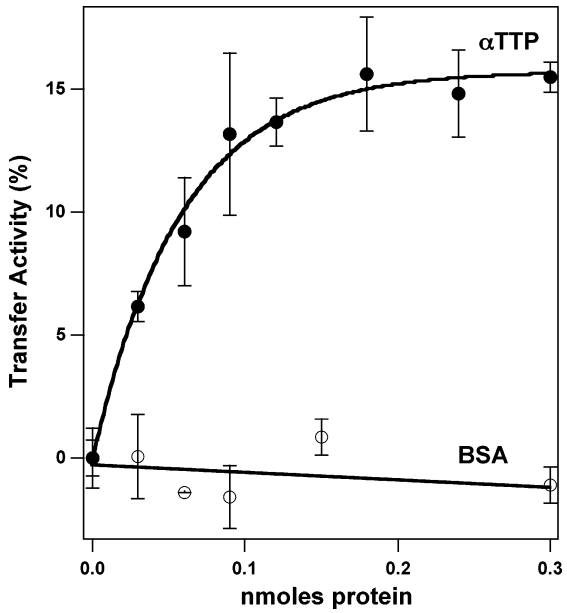

FIGURE 1: Recombinant TTP is competent in stimulating intermembrane tocopherol transfer. The indicated amount of purified protein was incubated with liposomes containing [^{14}C]-tocopherol and with mitochondria as described in Materials and Methods. After 30 min at 37 °C, mitochondria were centrifuged and radioactivity was measured in the supernatant. Transfer activity was calculated as described in Materials and Methods. A parallel experiment was carried out using commercial bovine serum albumin (open circles; Sigma Chemical Co., fatty acid free, purity > 99%).

tion of human TTP. We demonstrated that recombinant TTP, like the native liver protein, is capable of saturable, reversible, and high-affinity binding of tocopherol (*22*, *24*, *26*). Moreover, the ligand binding profile of recombinant TTP, like that of the native enzyme, closely parallels the biological potencies demonstrated by different vitamin E derivatives (*1*). Furthermore, the recombinant protein is as efficient in catalyzing tocopherol transfer as both the purified liver enzyme (*22*) and the wild-type protein overexpressed in cultured COS7 cells (*27*). Taken together, these observations support the notion that TTP is a critical mediator of vitamin E's biological activities (*28*, *29*). To better characterize the recombinant TTP protein, we assayed its ability to catalyze the transfer of tocopherol from liposomes to mitochondrial membranes. This activity of TTP is generally believed to be at the basis of the protein's function, and served as the purification criterion during the isolation of the native enzyme (*26*). As is shown in Figure 1, inclusion of purified recombinant TTP in assay mixtures leads to a marked facilitation of intermembrane transfer of tocopherol. The activity is dose-dependent, saturable, and appears to be TTP-specific, since a nonrelated protein (serum albumin) is ineffective in this assay. We conclude that, like the native enzyme, recombinant TTP is capable of tocopherol binding and transfer in vitro.

Tocopherol Binding and Transfer Activities of TTP Mutants. To gain molecular-level understanding of TTP's involvement in vitamin E biology, we compared the biochemical activities exhibited by the wild-type protein to those of substitution variants found in some human AVED patients (Table 1). The wild-type human TTP gene was utilized as a template for site-directed mutagenesis, and the resultant mutants were overexpressed in *E. coli* and purified to homogeneity (see Material and Methods). We first determined the affinity of each TTP variant for *RRR*-α-tocopherol using a Lipidex-based assay (*1*, *24*). Representative binding data are summarized in Table 2. All proteins tested bound α-tocopherol reversibly, stoichiometrically, and with a reasonably high affinity (<125 nM[2]). The R59W and R221W mutations significantly weakened TTP's affinity toward tocopherol (ca. 5-fold increase in dissociation constant), in agreement with the severe pathology associated with these amino acid substitutions. At least in the case of the R59W mutation, it appears that the functional perturbation may stem from alterations in the protein fold, since TTP molecules bearing more conservative mutations of the Arg^{59} residue (i.e., the R59K and R59A variants) have only minor effects on TTP's affinity for tocopherol (Table 2). All other AVED mutants exhibit affinities for *RRR*-α-tocopherol that we consider similar to that of the wild-type protein (<75 nM).

Next, we compared the TTP variants with respect to their ability to catalyze the time-dependent transfer of radiolabeled tocopherol from unilamellar vesicles to mitochondria. Kinetic data are shown in Figure 2 and summarized in Table 2. We found that all tested TTP proteins significantly facilitate the tocopherol transfer reaction. However, the TTP variants associated with the severe phenotype of the AVED syndrome

Table 1: Amino Acid Substitution Mutations Found in Human AVED Patients and Studies in This Report

nucleotide change	amino acid change	homologous residues in TTP, CRALBP, Sec14[a]	pathology	ref
C175T	R59W	R,R,R	severe	*40*
G421A	E141K	E,E,E	severe	*40*
C661T	R221W	R,R,K	severe	*40*
G358A	A120T	S,E,G	mild	*40*
T303G	H101Q	H,P,P	mild	*27*, *41*
G575A	R192H	R,K,G	mild	*40*, *42*

[a] Homologous residues determined from ClustalW alignment (*1*).

Table 2: Biochemical Characteristics of TTP Variants

TTP variant	K_d (nM)[a]	$t_{1/2}$ (min)[b]
wild-type	36.1 ± 5	5.9 ± 1.1
R59W	123.2 ± 11	11.8 ± 0.6
R59K	68.6 ± 6.8	6.6 ± 0.8
R59A	52.7 ± 4.3	6.4 ± 1.1
E141K	76.4 ± 8	15.7 ± 3.8
R221W	86.1 ± 11	17.3 ± 1.6
H101Q	63.4 ± 3.5	6.2 ± 1.2
A120T	70 ± 3.8	6.0 ± 0.9
R192H	40.9 ± 3.7	6.2 ± 0.5

[a] Affinities were determined using the Lipidex-based radioactive binding assay as described in Materials and Methods. At least three measurements were carried out using two or more protein preparations [b] Time-dependent tocopherol transfer was monitored as described in Figure 3. Raw data were fit to a single first-order process and the extracted half-life values are reported here.

[2] In light of the significant ligand promiscuity exhibited by members of the CRAL-TRIO family, it is instructive to define a numerical range of dissociation constants that are considered indicative of "high-affinity". We note that the CRALBP and Sec14 proteins (that are not considered to bind vitamin E under physiological conditions) bind tocopherol with a >400 nM affinity (*1*). Therefore, K_d values determined here for AVED mutants of TTP are indicative of specific and significant affinity for this ligand.

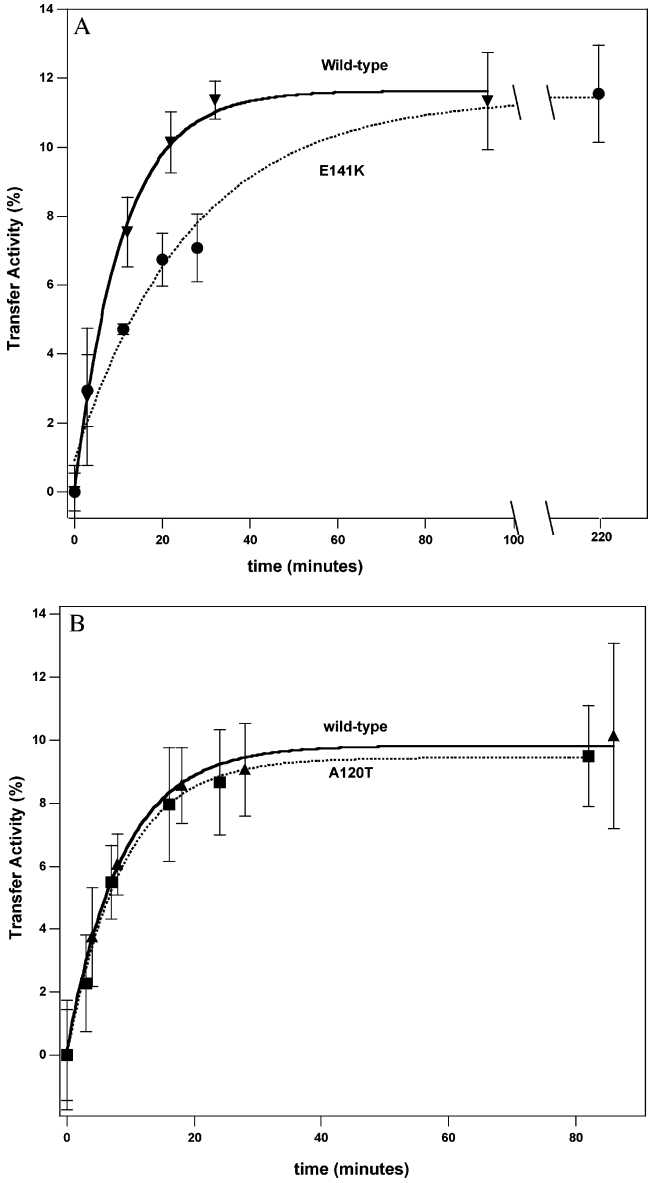

FIGURE 2: Tocopherol transfer activity of wild-type and representative mutant TTP. Time-dependent tocopherol transfer was monitored in the presence of 1.0 μg of the indicated purified TTP protein at 25 °C. Raw data (symbols) were fitted to a single first-order process (lines). Shown are representative data for the wild-type protein (solid triangles) as well as a severe (E141K; solid circles; panel A) and a mild (A120T; solid squares; panel B) AVED variant of TTP.

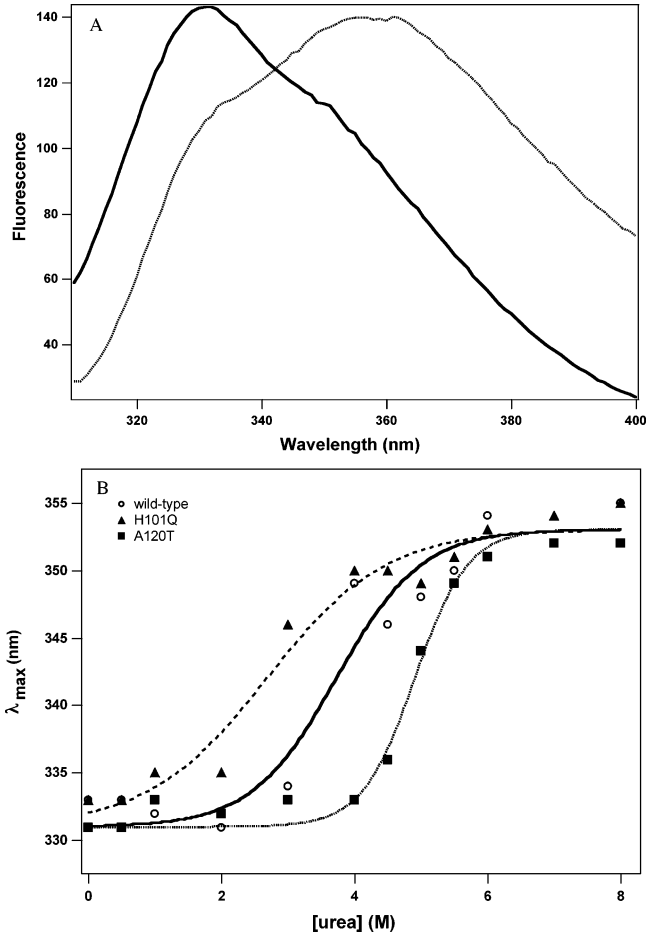

FIGURE 3: Urea-induced denaturation of TTP proteins. (A) The fluorescence emission spectrum of TTP in the absence (solid line) and presence (dotted line) of 8 M urea. Protein (0.5 μM) was excited at 294 nm, and emission spectra collected with a 3-nm resolution. (B) Urea-induced unfolding of TTP variants. Emission spectra were collected at the indicated urea concentrations, and emission maxima (λ_{max}) determined.

exhibit a kinetic impairment in this assay. Thus, TTP proteins harboring the R59W, E141K, and R221W mutations exhibited a 2−3-fold increase in the observed half-life for the protein-facilitated transfer reaction. On the other hand, the H101Q, A120T, and R192H mutants, associated with milder phenotypes of the AVED syndrome, are very similar to the wild-type protein in their ability to stimulate intermembrane transfer of tocopherol (Figure 3B, Table 2).

Conformational Integrity of TTP Mutants. The AVED-affected residues studied here are not in direct contact with the bound tocopherol in TTP's ligand-binding pocket. Indeed, we find that mutations of these residues lead to only a limited effect on ligand binding affinity (Table 2). We thus set out to assess whether these AVED mutations affect TTP by inducing a conformation that renders the protein unstable (it may be worth noting in this regard that we noticed marked variations among the TTP variants with respect to their expression levels in bacteria; see Materials and Methods). We examined the stability of the TTP variants by measuring their denaturant-induced unfolding patterns. We thus monitored the intrinsic fluorescence of the TTP proteins, in buffers containing increasing concentrations of urea (Figure 3). In the presence of high concentrations (>5 M) of urea, TTP's maximal emission spectrum undergoes a marked red shift from 333 to 355 nm (Figure 3A). Titrations of the unfolding process by using different urea concentrations reveal a smooth curve reflecting the transition between the folded and unfolded state (Figure 3B). Similar urea-induced spectral changes are observed with all AVED mutants of TTP. Importantly, mutations that impart the severe, early-onset pathology show unfolding profiles similar to those of the wild-type protein (Table 3). However, the AVED mutations associated with the milder form of the disease exhibited significantly different denaturation profiles. As compared to the wild-type TTP that displayed an unfolding midpoint of 3.8 M, the H101Q mutant appears to be significantly less stable, with a midpoint urea concentration for unfolding of 2.7 M. On the other hand, the A120T and R192H mutants appear to be more resistant to denaturation by urea, showing unfolding at higher urea concentrations (midpoint urea concentration = 4.9 M).

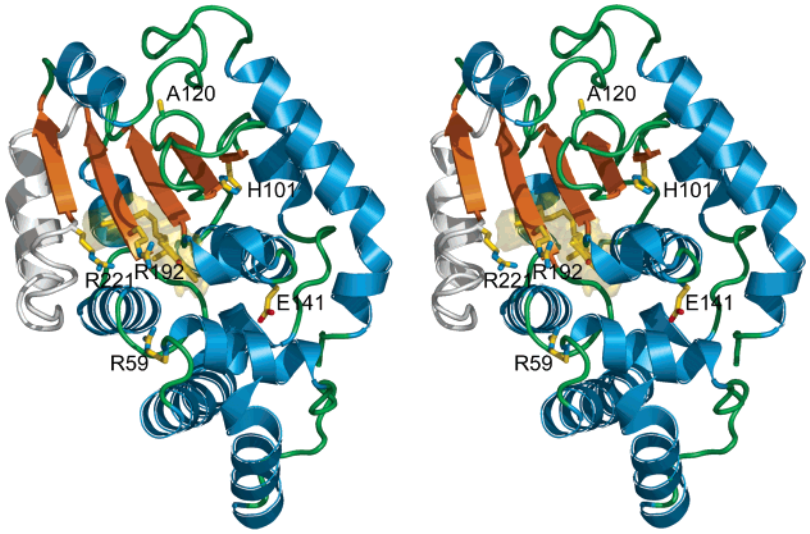

FIGURE 4: Stereodrawing of the three-dimensional structure of TTP. Bound RRR-α-tocopherol is shown as stick model in yellow. The cavity defined by the ligand-binding pocket of TTP is shown in orange. AVED-associated missense mutations are depicted as stick models in yellow. The lipid-exchange loop (residues 198−221) is shown in light gray. Replotted after Meier et al (*30*).

Table 3: Urea-Induced Unfolding of Wild-Type TTP and AVED Mutants

TTP variant	[Urea] C_{50} (M)[a]
wild-type	3.8
R59W	3.4
E141K	3.4
R221W	3.6
H101Q	2.7
A120T	4.9
R192H	4.9

[a] Shown are the urea concentrations required for half-maximal change (C_{50}) in emission wavelength maximum. Results are representative of two experiments. Fluorescence emission scans were collected as described in Figure 3 (excitation = 294 nm).

Structural Impact of AVED Mutations. It is informative to evaluate the functional implications of the AVED mutations studied here in the context of the recently solved crystal structure of TTP (*30*, *31*). The mutated residues are highlighted in TTP's three-dimensional structure in Figure 4. As can be seen, the mutations are scattered throughout the CRAL-TRIO fold, and none of the affected residues is in close direct contact (<6 Å) with the bound tocopherol. As shown in Figure 4, two of six missense mutations (E141K and H101Q) are located toward the interior of TTP. The E141K mutation on one side of helix 9 abolishes the hydrogen bond with Y73, while the H101Q mutation interferes with two hydrogen bonds: one with the backbone carbonyl of I116, and another with the hydroxyl group of T139 located on the other side of helix 9. Thus, it appears that helix 9 has a critical role in stabilizing the CRAL-TRIO fold of TTP. Accordingly, the H101Q substitution is accompanied by a marked increase in TTP's sensitivity to urea (Table 3). The other four AVED-associated mutations in TTP, namely, R59W, R221W, R192H, and A120T, are located at the protein's surface. The three arginines form a highly positively charged surface patch (see Figure 4) that is extended by additional basic residues (R57, R68, R151, K155, K190, and K217). It has been reported that residues R59W, R221W, and R192H surround the exit of a water-filled "channel" connecting the cavity of the tocopherol-binding pocket with the bulk solvent. In two of these missense mutations, namely, R59W and R221W, a highly conserved arginine residue is replaced by a hydrophobic tryptophan, while in the case of the R192H mutation a residue of intermediate polarity replaces R192. Mutation of Arg^{59} to a tryptophan leads to only a modest change in the protein's susceptibility to urea, decreasing the midpoint concentration by 0.4 M (Table 3). Similarly, conservative substitutions of this residue (R59K and R59A) enhance protein resistance to urea by a similar magnitude (data not shown). The slight impact of Arg^{59} substitutions on urea sensitivity suggests that this residue may not be central for maintaining the protein's overall integrity. In the A120T mutation, a potential hydrogen bond donor replaces the methyl side chain of A120 at the protein surface. We observe that the A120T and R192H mutations render the protein more resistant to urea-induced unfolding (Table 3).

DISCUSSION

Multiple lines of evidence point at TTP as a critical factor in maintenance of vitamin E homeostasis in mammals. The protein was initially isolated as a tocopherol binding protein in soluble liver extracts (*32*). The purified protein was then shown to exhibit specific high-affinity binding to α-tocopherol, and to facilitate the transfer of tocopherol between membranes (*20*, *33*). Furthermore, TTP's affinity for various tocols closely parallels the biological potencies of these vitamin E derivatives (*1*, *22*). Humans carrying mutations in the TTP gene display dramatic reduction in circulating tocopherol levels, together with a number of neuropathological symptoms typical of vitamin E deficiency, and termed ataxia with vitamin E deficiency (AVED, *34*). Recently, a number of groups reported the targeted disruption of the TTP gene in mice (*15*−*18*). As expected, $TTP^{-/-}$ mice exhibit very low levels of tocopherol in plasma and tissues, as well as the neuropathological hallmarks of AVED. If applied in time, supplementation with vitamin E can alleviate progression of the AVED disease in both human patients (*35*) and in the $TTP^{-/-}$ mice (*18*).

To gain better understanding into the role of TTP in vitamin E biology, we compared the biochemical properties

of TTP variants that are found in human AVED patients. We reasoned that since each of the six TTP missense mutations leads to perturbations in vitamin E homeostasis the mutated proteins could offer important insights into the mechanism(s) by which TTP regulates tocopherol secretion. Thus, we characterized the in vitro tocopherol binding and transfer activities of these proteins. Purified recombinant wild-type TTP exhibited a high affinity for tocopherol (K_d = 36 nM, Table 2 and ref *1*), and a pronounced ability to facilitate the transfer of tocopherol between membranes (half-time for the catalyzed transfer reaction of ~6 min). All TTP mutants assessed in this study exhibited measurable activity in these assays. We find that variants associated with the severe (early-onset) version of the AVED pathology are impaired in both binding and transfer activities. Thus, the R59W, E141K, and R221W mutants exhibit a 2−3-fold reduction in their ligand binding activities (K_d for *RRR*-α-tocopherol 76−123 nM). Similarly, these mutated proteins showed a 2−3-fold impairment in their ability to catalyze tocopherol transfer between lipid membranes. TTP variants associated with the milder (late-onset) forms of AVED, i.e., the H101Q, A120T, and R192H substitutions, on the other hand, exhibit a high overall affinity for tocopherol, in the range displayed by the wild-type protein. In intermembrane tocopherol transfer assays, the mild AVED TTP proteins were strikingly similar to the wild-type protein.

Since the functional manifestation of the AVED mutations was milder than anticipated, we wondered whether the mutated proteins are compromised in their folding and gross structural integrity. To address this possibility, we employed urea-induced denaturation experiments, where protein unfolding was assessed by monitoring intrinsic fluorescence (Figure 3, Table 3). All proteins tested exhibited a well-defined urea-induced spectral transition from a folded state (λ_{max} emission = 332 ± 4 nm) to an unfolded state (λ_{max} emission = 355 ± 4 nm). The R59W, E141K, and R221W mutants displayed urea denaturation profiles similar to the wild-type protein. Surprisingly, only the "mild" AVED mutations differed from the wild-type protein in their unfolding patterns. Specifically, the R192H and the A120T mutants were more resistant than the wild-type protein, as evident from their attenuated response to denaturation by urea. Conformation of the H101Q mutant, on the other hand, appears to be less stable, as this protein unfolds at lower urea concentration. This increased lability could explain why the H101Q mutant was reported by another group to be completely inactive (*27*). The R59W, R221W, and R192H mutations in TTP affect a positive "patch" at the protein's surface. Interestingly, this feature is conserved among CRAL-TRIO members, and mutations in homologous residues were reported to affect function. Specifically, the K239A mutation in Sec14p is defective in phosphatidylinositol transfer (*36*), and the R233W mutation in CRALBP is associated with retinal degeneration (*37*). It is tempting to speculate that the positive surface formed by these conserved residues is critical for "docking" the protein to the negatively charged headgroups of membrane lipids.

Taken together, our data suggest that the physiological phenotypes associated with the AVED mutations studied here do *not* stem from perturbations of TTP's overall structural integrity. However, the possibility still exists that the mutated proteins are less stable in vivo. Direct measurements of the synthesis/degradation rates of the mutated TTP proteins in cultured cells will directly address this possibility.

How does TTP regulate vitamin E homeostasis? It is generally assumed the protein's activity in vivo is reflected in the in vitro transfer assay originally used to isolate the protein. Our data suggest that there is only a weak correlation between TTP's activity in the intermembrane tocopherol transfer assay, and the protein's functionality in vivo. Specifically, the R192H, A120T, and H101Q AVED mutants are remarkably similar to the wild-type protein with respect to their functionality in the in vitro intermembrane transfer assay. In vivo, however, these mutations lead to pronounced perturbations of vitamin E status. Similarly, TTP mutations associated with the severe, early-onset form of AVED (i.e., the R59W, E141K, and R221W variants) exhibit a surprisingly mild (2−3-fold) kinetic impairment in their ability to catalyze tocopherol transfer. An immediate question arises: how can such a benign attenuation of protein activity lead to the observed pathology in vivo? One possible explanation is that tocopherol levels are under very tight regulation. In this case, minor changes in tocopherol processing could greatly impact tocopherol homeostasis, and lead to the observed pathologies. In support of this notion are reports of multiple mechanisms by which tocopherols affect normal cell function (*38*). An alternative explanation of our observations is that in vivo the function of TTP involves additional, yet to be described, biochemical activity(ies). If such is the case, it is impossible to fully recapitulate TTP's function in vitro using only the signature assays of tocopherol binding and intermembrane transfer.

It is tempting to speculate that the mechanisms underlying TTP-dependent tocopherol secretion involve other cellular components, possibly proteins. In support of this notion is the observation that in Tangier disease cells, in which the lipid/cholesterol transporter ABC-A1 is mutated, tocopherol secretion is markedly impaired (*39*). We anticipate that the availability of the TTP mutants described here will facilitate testing such hypotheses, and the arrival at a better picture of TTP's biological function.

REFERENCES

1. Panagabko, C., Morley, S., Hernandez, M., Cassolato, P., Gordon, H., Parsons, R., Manor, D., and Atkinson, J. (2003) Ligand specificity in the CRAL-TRIO protein family. *Biochemistry 42*, 6467−6474.
2. Evans, H. M., and Bishop, K. S. (1922) On the existence of a hitherto unrecognized dietary factor essential for reproduction. *Science 56*, 650−651.
3. Burton, G. W., Cheeseman, K. H., Doba, T., Ingold, K. U., and Slater, T. F. (1983) Vitamin E as an antioxidant in vitro and in vivo. *Ciba Found. Symp. 101*, 4−18.
4. Burton, G. W., and Traber, M. G. (1990) Vitamin E: antioxidant activity, biokinetics, and bioavailability. *Annu. Rev. Nutr. 10*, 357−382.
5. Horwitt, M. K., Elliott, W. H., Kanjananggulpan, P., and Fitch, C. D. (1984) Serum concentrations of alpha-tocopherol after ingestion of various vitamin E preparations. *Am. J. Clin. Nutr. 40*, 240−245.
6. Leth, T., and Sondergaard, H. (1983) Biological activity of all-rac-α-tocopherol and RRR-α-tocopherol determined by three different rat bioassays. *Int. J. Vitam. Nutr. Res. 53*, 297−311.
7. Ames, S. R. (1979) Biopotencies in rats of several forms of alpha-tocopherol. *J. Nutr. 109*, 2198−2204.
8. Burton, G. W., Traber, M. G., Acuff, R. V., Walters, D. N., Kayden, H., Hughes, L., and Ingold, K. U. (1998) Human plasma and tissue alpha-tocopherol concentrations in response to supple-

mentation with deuterated natural and synthetic vitamin E. *Am. J. Clin. Nutr. 67*, 669−684.
9. Behrens, W. A., and Madere, R. (1986) Alpha- and gamma tocopherol concentrations in human serum. *J. Am. Coll. Nutr. 5*, 91−96.
10. Traber, M. G., and Kayden, H. J. (1989) Alpha-tocopherol as compared with gamma-tocopherol is preferentially secreted in human lipoproteins. *Ann. N. Y. Acad. Sci. 570*, 95−108.
11. Traber, M. G., and Arai, H. (1999) Molecular mechanisms of vitamin E transport. *Annu. Rev. Nutr. 19*, 343−355.
12. Copp, R. P., Wisniewski, T., Hentati, F., Larnaout, A., Ben Hamida, M., and Kayden, H. J. (1999) Localization of alpha-tocopherol transfer protein in the brains of patients with ataxia with vitamin E deficiency and other oxidative stress related neurodegenerative disorders. *Brain Res. 822*, 80−87.
13. Traber, M. G., Sokol, R. J., Kohlschutter, A., Yokota, T., Muller, D. P., Dufour, R., and Kayden, H. J. (1993) Impaired discrimination between stereoisomers of alpha-tocopherol in patients with familial isolated vitamin E deficiency. *J. Lipid Res. 34*, 201−210.
14. Arita, M., Sato, Y., Miyata, A., Tanabe, T., Takahashi, E., Kayden, H. J., Arai, H., and Inoue, K. (1995) Human alpha-tocopherol transfer protein: cDNA cloning, expression and chromosomal localization. *Biochem. J. 306*, 437−443.
15. Terasawa, Y., Ladha, Z., Leonard, S. W., Morrow, J. D., Newland, D., Sanan, D., Packer, L., Traber, M. G., and Farese, R. V., Jr. (2000) Increased atherosclerosis in hyperlipidemic mice deficient in alpha-tocopherol transfer protein and vitamin E. *Proc. Natl. Acad. Sci. U.S.A. 97*, 13830−4.
16. Jishage, K., Arita, M., Igarashi, K., Iwata, T., Watanabe, M., Ogawa, M., Ueda, O., Kamada, N., Inoue, K., Arai, H., and Suzuki, H. (2001) Alpha-tocopherol transfer protein is important for the normal development of placental labyrinthine trophoblasts in mice. *J. Biol. Chem. 276*, 1669−1672.
17. Leonard, S. W., Terasawa, Y., Farese, R. V., Jr., and Traber, M. G. (2002) Incorporation of deuterated RRR− or all-rac-alpha-tocopherol in plasma and tissues of alpha-tocopherol transfer protein-null mice. *Am. J. Clin. Nutr. 75*, 555−560.
18. Yokota, T., Igarashi, K., Uchihara, T., Jishage, K., Tomita, H., Inaba, A., Li, Y., Arita, M., Suzuki, H., Mizusawa, H., and Arai, H. (2001) Delayed-onset ataxia in mice lacking alpha -tocopherol transfer protein: model for neuronal degeneration caused by chronic oxidative stress. *Proc. Natl. Acad. Sci. U.S.A. 98*, 15185−15190.
19. Hosomi, A., Goto, K., Kondo, H., Iwatsubo, T., Yokota, T., Ogawa, M., Arita, M., Aoki, J., Arai, H., and Inoue, K. (1998) Localization of alpha-tocopherol transfer protein in rat brain. *Neurosci. Lett. 256*, 159−162.
20. Mowri, H., Nakagawa, Y., Inoue, K., and Nojima, S. (1981) Enhancement of the transfer of alpha-tocopherol between liposomes and mitochondria by rat-liver protein(s). *Eur. J. Biochem. 117*, 537−542.
21. Verdon, C. P., and Blumberg, J. B. (1988) An assay for the alpha-tocopherol binding protein mediated transfer of vitamin E between membranes. *Anal. Biochem. 169*, 109−120.
22. Hosomi, A., Arita, M., Sato, Y., Kiyose, C., Ueda, T., Igarashi, O., Arai, H., and Inoue, K. (1997) Affinity for alpha-tocopherol transfer protein as a determinant of the biological activities of vitamin E analogs. *FEBS Lett. 409*, 105−108.
23. Arita, M., Nomura, K., Arai, H., and Inoue, K. (1997) Alpha-tocopherol transfer protein stimulates the secretion of alpha-tocopherol from a cultured liver cell line through a brefeldin A-insensitive pathway. *Proc. Natl. Acad. Sci. U.S.A. 94*, 12437−12441.
24. Panagabko, C., Morley, S., Neely, S., Lei, H., Manor, D., and Atkinson, J. (2002) Expression and refolding of recombinant human alpha-tocopherol transfer protein capable of specific alpha-tocopherol binding. *Protein Expr. Purif. 24*, 395−403.
25. Johnson, L. W., and Zilversmit, D. B. (1975) Catalytic properties of phospholipid exchange protein from bovine heart. *Biochim. Biophys. Acta 375*, 165−175.
26. Sato, Y., Hagiwara, K., Arai, H., and Inoue, K. (1991) Purification and characterization of the alpha-tocopherol transfer protein from rat liver. *FEBS Lett. 288*, 41−45.
27. Gotoda, T., Arita, M., Arai, H., Inoue, K., Yokota, T., Fukuo, Y., Yazaki, Y., and Yamada, N. (1995) Adult-onset spinocerebellar dysfunction caused by a mutation in the gene for the alpha-tocopherol-transfer protein. *N. Engl. J. Med. 333*, 1313−1318.
28. Kaempf-Rotzoll, D. E., Traber, M. G., and Arai, H. (2003) Vitamin E and transfer proteins. *Curr. Opin. Lipidol. 14*, 249−254.
29. Blatt, D. H., Leonard, S. W., and Traber, M. G. (2001) Vitamin E kinetics and the function of tocopherol regulatory proteins. *Nutrition 17*, 799−805.
30. Meier, R., Tomizaki, T., Schulze-Briese, C., Baumann, U., and Stocker, A. (2003) The molecular basis of vitamin E retention: structure of human alpha-tocopherol transfer protein. *J. Mol. Biol. 331*, 725−734.
31. Min, K. C., Kovall, R. A., and Hendrickson, W. A. (2003) Crystal Structure of human alpha-tocopherol transfer protein bound to its ligand: implications for ataxia with vitamin E deficiency. *Proc. Natl. Acad. Sci. U.S.A. 100*, 14713−14718.
32. Catignani, G. L. (1975) An alpha-tocopherol binding protein in rat liver cytoplasm. *Biochem Biophys Res. Commun. 67*, 66−72.
33. Murphy, D. J., and Mavis, R. D. (1981) Membrane transfer of alpha-tocopherol. Influence of soluble alpha-tocopherol-binding factors from the liver, lung, heart, and brain of the rat. *J. Biol. Chem. 256*, 10464−10468.
34. Ouahchi, K., Arita, M., Kayden, H., Hentati, F., Ben Hamida, M., Sokol, R., Arai, H., Inoue, K., Mandel, J. L., and Koenig, M. (1995) Ataxia with isolated vitamin E deficiency is caused by mutations in the alpha-tocopherol transfer protein. *Nat. Genet. 9*, 141−145.
35. Gordon, N. (2001) Hereditary vitamin-E deficiency. *Dev. Med. Child Neurol. 43*, 133−135.
36. Phillips, S. E., Sha, B., Topalof, L., Xie, Z., Alb, J. G., Klenchin, V. A., Swigart, P., Cockcroft, S., Martin, T. F., Luo, M., and Bankaitis, V. A. (1999) Yeast Sec14p deficient in phosphatidylinositol transfer activity is functional in vivo. *Mol. Cell 4*, 187−197.
37. Golovleva, I., Bhattacharya, S., Wu, Z., Shaw, N., Yang, Y., Andrabi, K., West, K. A., Burstedt, M. S., Forsman, K., Holmgren, G., Sandgren, O., Noy, N., Qin, J., and Crabb, J. W. (2003) Disease-causing mutations in the cellular retinaldehyde binding protein tighten and abolish ligand interactions. *J. Biol. Chem. 278*, 12397−12402.
38. Brigelius-Flohe, R., and Traber, M. G. (1999) Vitamin E: function and metabolism. *FASEB J. 13*, 1145−1155.
39. Oram, J. F., Vaughan, A. M., and Stocker, R. (2001) ATP-binding cassette transporter A1 mediates cellular secretion of alpha-tocopherol. *J. Biol. Chem. 276*, 39898−39902.
40. Cavalier, L., Ouahchi, K., Kayden, H. J., Di Donato, S., Reutenauer, L., Mandel, J. L., and Koenig, M. (1998) Ataxia with isolated vitamin E deficiency: heterogeneity of mutations and phenotypic variability in a large number of families. *Am. J. Hum. Genet. 62*, 301−310.
41. Yokota, T., Shiojiri, T., Gotoda, T., Arita, M., Arai, H., Ohga, T., Kanda, T., Suzuki, J., Imai, T., Matsumoto, H., Harino, S., Kiyosawa, M., Mizusawa, H., and Inoue, K. (1997) Friedreich-like ataxia with retinitis pigmentosa caused by the His101Gln mutation of the alpha-tocopherol transfer protein gene. *Ann. Neurol. 41*, 826−832.
42. Hentati, A., Deng, H. X., Hung, W. Y., Nayer, M., Ahmed, M. S., He, X., Tim, R., Stumpf, D. A., Siddique, T., and Ahmed, A. (1996) Human alpha-tocopherol transfer protein: gene structure and mutations in familial vitamin E deficiency. *Ann. Neurol. 39*, 295−300.

BI0363073