Influence of major structural features of tocopherols and tocotrienols on their ω-oxidation by tocopherol-ω-hydroxylase

Timothy J. Sontag and Robert S. Parker[1]

Division of Nutritional Sciences, Cornell University, Ithaca, NY 14850

Abstract Human cytochrome P450 4F2 (CYP4F2) catalyzes the initial ω-hydroxylation reaction in the metabolism of tocopherols and tocotrienols to carboxychromanols and is, to date, the only enzyme shown to metabolize vitamin E. The objective of this study was to characterize this activity, particularly the influence of key features of tocochromanol substrate structure. The influence of the number and positions of methyl groups on the chromanol ring, and of stereochemistry and saturation of the side chain, were explored using HepG2 cultures and microsomal reaction systems. Human liver microsomes and microsomes selectively expressing recombinant human CYP4F2 exhibited substrate activity patterns similar to those of HepG2 cells. Although activity was strongly associated with substrate accumulation by cells or microsomes, substantial differences in specific activities between substrates remained under conditions of similar microsomal membrane substrate concentration. Methylation at C5 of the chromanol ring was associated with markedly low activity. Tocotrienols exhibited much higher V_{max} values than their tocopherol counterparts. Side chain stereochemistry had no effect on ω-hydroxylation of α-tocopherol (α-TOH) by any system. Kinetic analysis of microsomal CYP4F2 activity revealed Michaelis-Menten kinetics for α-TOH but allosteric cooperativity for other vitamers, especially tocotrienols. Additionally, α-TOH was a positive effector of ω-hydroxylation of other vitamers. These results indicate that CYP4F2-mediated tocopherol-ω-hydroxylation is a central feature underlying the different biological half-lives, and therefore biopotencies, of the tocopherols and tocotrienols.—Sontag, T. J., and R. S. Parker. Influence of major structural features of tocopherols and tocotrienols on their ω-oxidation by tocopherol-ω-hydroxylase. *J. Lipid Res.* 2007. **48:** 1090–1098.

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Vitamin E is the generic term for the tocopherols and tocotrienols, which differ in the number and positions of methyl groups around the chromanol ring and in the saturation and stereochemistry of the phytyl tail (**Fig. 1**).

These subtle structural variations are associated with substantial differences in their biopotency in vivo. Although α-tocopherol (α-TOH) has been studied most intensively, recent research has also suggested important roles for the non-α vitamers of vitamin E. Bioactivities of tocopherols and tocotrienols include the more familiar trapping of radical species, including nitric oxide (1–9), as well as more provocative activities, such as the regulation of gene transcription (10–13), anti-inflammation (14), inhibition of cholesterol synthesis (15), and apoptosis (16–18).

The superior activity of RRR-α-TOH in vivo appears to result from its superior retention in the body relative to other forms of vitamin E (19, 20). Although an important factor involved in this selectivity is the hepatic α-tocopherol transfer protein (α-TTP) (21–24), differential rates of postabsorptive metabolism of the tocopherols and tocotrienols to water-soluble metabolites appear to be ultimately responsible for the dramatic differences in biological half-lives between the various vitamers. The tocopherols and tocotrienols are metabolized to water-soluble urinary metabolites with the side chain shortened to the 3′ carbon (25–29). We demonstrated that this occurs by an initial ω-hydroxylation at a terminal methyl group of the hydrophobic side chain, followed by sequential β-oxidation to the 3′ carboxychromanol, or 2,7,8-trimethyl-2-(β-carboxyethyl)-6-hydroxychroman (CEHC) (30). The intermediates in this pathway have been identified for both the tocopherols and tocotrienols (30, 31). We further demonstrated that the initial ω-hydroxylation step in this pathway is catalyzed by a member of the cytochrome P450 family of enzymes, cytochrome P450 4F2 (CYP4F2), previously characterized as a leukotriene B4 ω-hydroxylase (32). The activity of this enzyme was severalfold greater toward γ-TOH than toward α-TOH (30). The pathway appears to be an important regulator of vitamer status in vivo, as the inhibition of
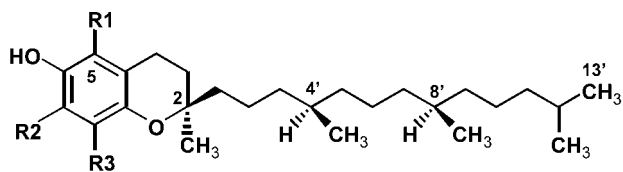
		R1	R2	R3
Tri-methyl	α	CH₃	CH₃	CH₃
Di-methyl	γ	H	CH₃	CH₃
Di-methyl	β	CH₃	H	CH₃
Di-methyl	ε	CH₃	CH₃	H
Mono-methyl	δ	H	H	CH₃
Non-methyl	τ	H	H	H

Tocopherols = no double bonds in side chain
All rac = racemic at carbons 2, 4', and 8'
Tocotrienols = double bonds at carbons 3'-4', 7'-8', and 11'-12'

Fig. 1. Vitamin E structure, depicting key features of the chromanol ring and side chain.

tocopherol-ω-hydroxylation by sesame lignans in rats increases plasma levels of γ-TOH to levels approaching those of α-TOH (3).

Given the apparent important contribution of tocopherol-ω-hydroxylase activity to vitamin E status in vivo, we investigated the influence of structural variations among the vitamers of vitamin E on the activity of this enzyme. The effects of phenol ring methylation and of stereochemistry and unsaturation of the side chain were systematically determined in both cell culture and microsomal membrane models. Kinetic analyses were carried out to investigate potential substrate interactions and the role of tocopherol-ω-hydroxylase in the suppression effect of supplemental α-TOH on levels of other tocopherols in vivo (20, 33).

MATERIALS AND METHODS

Tocopherols were purchased from Fluka Biochemicals (Milwaukee, WI; RRR-γ-TOH), ACROS Organics, Fisher Scientific (Pittsburgh, PA; RRR-α-TOH), or Matreya, Inc. (rac-α-, rac-γ-, rac-β-, ε-TOH, and tocol). The tocotrienols were a gift from Volker Berl (BASF, Ludvigshafen, Germany). β-NADPH and NAD were purchased from Sigma Chemical Co. (St. Louis, MO). Pooled normal human liver microsomes, and insect cell microsomes (BD Supersomes) containing human CYP4F2, cytochrome P450 reductase, and cytochrome b₅ expressed using a baculovirus expression system, were purchased from BD Gentest, Inc. (Woburn, MA). Rat liver microsomes were prepared as described previously (30). Microsomes were stored at −70°C. HepG2 cells (C3A subclone CRL-10741) were purchased from the American Type Culture Collection (Manassas, VA). Primary bovine aortic endothelial cells and rat basophilic leukemia (RBL-2H3) cells were gifts from B. Pauli (Cornell University School of Veterinary Medicine) and B. Baird (Cornell University Department of Chemistry and Chemical Biology), respectively.

HepG2 cell culture

HepG2 cultures were maintained in DMEM containing NaHCO₃ and 10% FBS (U.S. Department of Agriculture certified; Mediatech, Inc., Herndon, VA) under standard cell culture conditions without antibiotics.

Substrate-enriched medium was prepared using an appropriate volume of each tocochromanol (5–30 mM solution in ethanol) added drop-wise to FBS while mixing. The FBS was stored at 4°C for a minimum of 4 h, then diluted 1:10 with DMEM. Final substrate concentrations were 25 μM, and ethanol concentrations were <0.85%. After 0–48 h of incubation with cells, the medium was collected and cells were washed and scraped into 0.9% NaCl. Medium and cells were stored at −20°C under argon until analyzed.

Microsomal reaction system

The microsomal reaction system consisted of 100 mM KH₂PO₄ buffer (pH 7.4) with 100 μg protein/ml pooled human or rat liver microsomes, or 20 pmol P450/ml CYP4F2 insect cell microsomes, and 0.5–1.0 mM NADPH + NAD. Substrate tocopherols and tocotrienols were added as a complex with 1% (w/v) fraction V BSA prepared as described previously (30). Microsomes were preincubated with the substrate-BSA complex at 37°C for 60 min to allow substrate to associate with membrane. Reactions were initiated immediately with the addition of NADPH + NAD either in the presence of any remaining exogenous substrate-BSA immediately after the preincubations or after centrifugal reisolation of substrate-preloaded membranes to remove unbound substrate. Substrate-preloaded membranes were isolated by centrifugation for 1 h at 100,000 g, washed and resuspended in reaction buffer, and used immediately. Microsome-associated substrate concentrations were determined as described below. Reactions were carried out for 0–40 min at 37°C and terminated by the addition of 100 μl of 3 N HCl and 2 volumes of cold absolute ethanol. No reaction products were observed in the absence of added NADPH.

Substrate and metabolite analyses

For the analysis of short-chain metabolites in cell culture, custom-synthesized deuterium-labeled d₉-α-CEHC was added to medium samples (3 ml), which were then acidified to pH 1.5 with 3 N HCl and extracted with ethyl acetate. Cell pellet or microsome suspensions were sonicated, acidified to pH 1.5 with 3 N HCl, and precipitated with 2 volumes of cold absolute ethanol. Long-chain metabolites and the parent tocopherol or tocotrienol were extracted from both cell suspensions and microsomal reaction systems with 1 volume of methyl-tert-butyl-ether and 8 volumes of hexane after the addition of d₉-α-TOH as an internal standard. Solvents were removed under a stream of N₂, and the residue was silylated with pyridine and N,O-bis-[trimethylsilyl]trifluoroacetamide containing 1% trimethylchlorosilane (Pierce Chemical Co., Rockford, IL) under N₂ at 70°C for 30 min.

GC-MS

A Hewlett-Packard 6890 gas chromatograph, coupled to a Hewlett-Packard 5872 mass selective detector operated in either selected ion or scan mode, was used for all analyses. The gas chromatograph was fitted with a Hewlett-Packard HP-1 methylsiloxane capillary column (30 m × 0.25 mm) and operated in split injection mode using helium as the carrier gas. For short-chain tocopherol metabolite analyses, the oven was programmed to ramp from 200°C (2 min hold) to 250°C at 7°C/min, followed by a 6 min hold at 250°C, then ramped to 280°C at 25°C/min, with a final hold at 280°C for 9 min. Long-chain metabolites and parent substrates were resolved isothermally at 280°C for 10 min.

Tocopherol and metabolite concentrations were determined using the appropriate deuterated internal standards. Cell- and microsome-associated substrates were expressed relative to unesterified cholesterol, an indicator of membrane mass.

Kinetic analysis

Kinetic constants were obtained using OriginLab Origin 7 software. Kinetic data were analyzed using a nonlinear curve fit method included in the software to yield best fit line and error values.

RESULTS

The comparative metabolism of the various forms of vitamin E was examined in hepatocyte culture and in various microsomal systems, including human liver microsomes and microsomes from insect cells in which recombinant human cytochrome P450 tocopherol-ω-hydroxylase (CYP4F2) was selectively expressed. Total flux through the ω-hydroxylation/β-oxidation pathway was expressed as the sum of the metabolites produced over the course of the experiment. Rates of metabolism of the vitamers were compared based on four features of the tocochromanol molecule depicted in Fig. 1: *a*) extent of methylation of the chromanol ring (α-, γ-, and δ-TOH and tocol, which contain three, two, one, and zero methyl groups respectively); *b*) positions of these methyl groups, focusing on the dimethyl tocopherols (γ-, β-, and ε-TOH, which lack a methyl group at positions 5, 7, and 8, respectively); *c*) stereochemistry of the side chain (RRR- vs. all-rac-α- and γ-TOH); and *d*) saturation of the side chain [α-, γ-, and δ-TOH vs. α-, γ-, and δ-tocotrienol (α-, γ-, and δ-T3)].

Tocochromanol metabolism in hepatocyte cell culture

HepG2/C3A hepatocytes, which do not express detectable α-TTP protein, were incubated with equimolar concentrations of the 11 vitamers individually for 48 h, and the generation of total metabolites was compared (**Fig. 2**). The hepatocytes displayed substrate discrimination based on the structural features described. Nonmethylated tocol and monomethylated δ-TOH were most effectively converted to their water-soluble urinary metabolites, followed by the dimethyl γ-TOH, whereas trimethyl α-TOH was poorly metabolized. The comparison of metabolism of the dimethyl tocopherols revealed a strong inhibitory effect of methylation at carbon 5 (β-TOH), whereas methylation at other positions (γ- and ε-TOH) were not as influential. RRR- and rac-α-TOH were metabolized at similarly low rates, whereas RRR-γ-TOH was metabolized more extensively than rac-γ-TOH. The structural feature contributing most to flux through the tocopherol-ω-oxidation pathway was unsaturation of the side chain. Each of the three tocotrienols was metabolized to a greater extent than its corresponding tocopherol.

Comparison of HepG2 substrate content over time revealed large differences in the accumulation of various vitamers. As illustrated in **Fig. 3**, decreased ring methylation increased the rate of tocopherol incorporation into
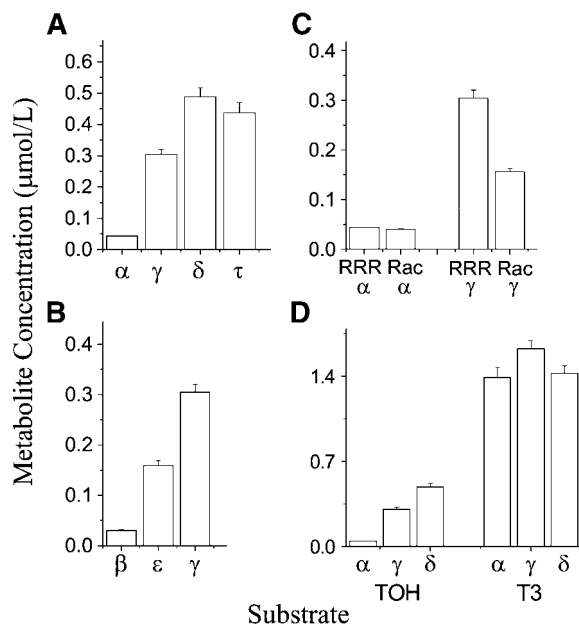

Fig. 2. Influence of tocochromanol structure on metabolism in HepG2 cultures. Confluent cultures were incubated for 48 h with 25 μM substrate, and total metabolite in the medium was quantified as described in Materials and Methods. Data are grouped by structural feature. A: Number of methyl groups. B: Methyl group position. C: Side chain stereochemistry. D: Side chain saturation. Error bars represent means ± SD.

cells. Tocol displayed the highest rate of cell incorporation. Tocotrienols were more rapidly taken up by cells than their corresponding tocopherols. There were no significant effects of methyl position or side chain stereochemistry on the rate of substrate uptake by cells. Similar accumulation differences were obtained when tested in the presence of sesamin, an inhibitor of tocopherol-ω-hydroxylase (30), and in primary bovine aortic endothelial cells or RBL-2H3 cells, neither of which metabolizes vitamin E (data not shown). Thus, although metabolism of tocochromanols may be influenced by differential accumulation, the pattern of accumulation is not materially influenced by metabolism, probably because only a small proportion of substrate ($<1\%$) is metabolized within the time course of the experiments. These data are consistent with reports of variation of vitamer uptake in other cell types (5, 34).

Organelle isolation assays revealed rapid distribution of vitamin E from the extracellular medium into mitochondria, here used as an indicator of the rate of vitamer movement to inner cellular membranes, where the P450 ω-hydroxylation likely occurs. After 2 h of incubation with 25 μM substrate, the tocotrienols were present in the mitochondrial fraction at levels >3-fold higher than those of the corresponding tocopherols (data not shown).

Microsomal metabolism of tocochromanols

The cell culture data strongly suggested structural discrimination by the enzyme(s) involved in the side chain
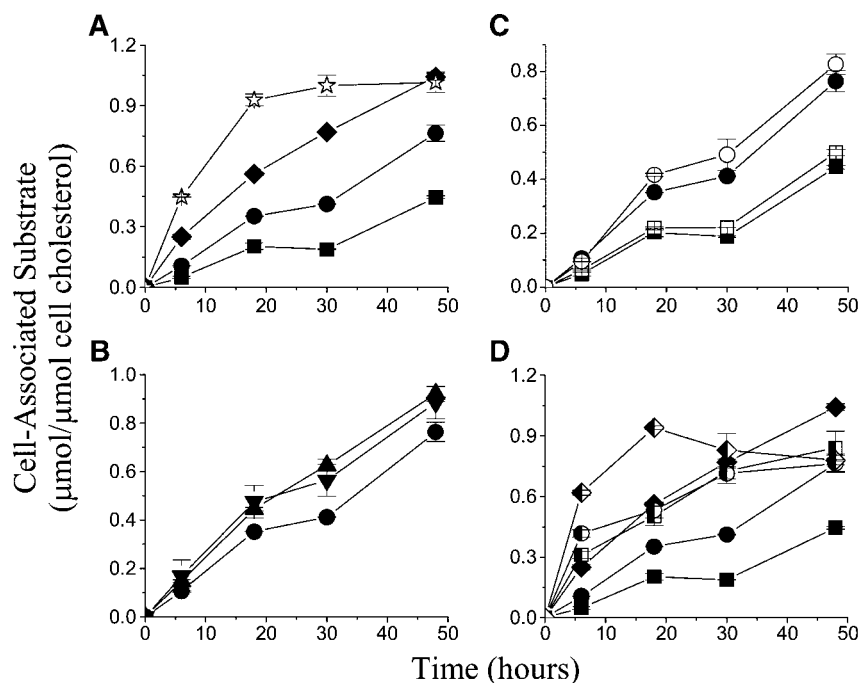
Fig. 3. Variation in the accumulation of different forms of vitamin E in HepG2 cells. Confluent cultures were incubated with 25 μM substrate and harvested at 6, 18, 30, and 48 h, and the amount of substrate associated with the cell was measured. Data are grouped by structural feature. A: Number of methyl groups. B: Methyl group position. C: Side chain stereochemistry. D: Side chain saturation. Closed squares, α-tocopherol (α-TOH); open squares, rac-α-TOH; closed circles, γ-TOH; open circles, rac-γ-TOH; closed triangles, β-TOH; inverted closed triangles, ε-TOH; closed diamonds, δ-TOH; open stars, tocol; half-closed/half-open squares, α-tocotrienol (α-T3); half-closed/half-open circles, γ-T3; half-closed/half-open diamonds, δ-T3. Error bars represent means ± SD.

truncation of the tocochromanol substrates. However, variation in cell uptake and distribution among vitamers confounded direct determination of the contribution of enzyme activity per se to the observed differential rates of metabolism. Therefore, we developed a microsomal assay in which substrates were presented directly to the microsomal membrane containing the cytochrome P450 complex.

Microsomal assays were conducted for each of the substrates using commercially available human liver microsomes or insect cell microsomes selectively expressing recombinant human liver CYP4F2. Preincubation of BSA-bound substrate with the microsomes consistently resulted in greater metabolite production than without preincubation, suggesting that the membrane-associated substrate was the pool upon which tocopherol-ω-hydroxylase drew (data not shown). As suspected from the cell culture results, the efficiency of incorporation of substrate into the microsomal membrane varied dramatically between substrates. This necessitated, for each substrate, the determination of the concentration of BSA-bound substrate used for preincubation that would yield a given substrate concentration in the reisolated microsomal membrane and the range of linearity of the relationship between added BSA-bound substrate and membrane substrate concentration.

Tocopherol-ω-hydroxylase activity of human liver microsomes was compared under two conditions. In both, microsomes were preincubated with concentrations of substrate

that had been determined previously to yield 100 nmol substrate/mg protein in the membrane in the reisolated microsomes. In the first condition, the microsomal reaction was carried out in the presence of both membrane-associated and unbound substrate. In the second condition, microsomes were reisolated by ultracentrifugation and the reaction was carried out in the presence of membrane-associated substrate only. The results showed a similar pattern of vitamer metabolism under both conditions (**Fig. 4A**). This occurred despite the wide range of total vitamer concentration present attributable to unbound substrate in the first condition (Fig. 4B). The overall activity under the first condition was higher, either because of continued uptake of aqueous (BSA-bound) vitamer into the membrane during the reaction or from a partial loss of enzyme activity during membrane reisolation under the second condition.

A time course study of metabolite production in human liver microsomes was conducted. Extrapolation of metabolite production to earlier time points indicated a transient initial "burst" of metabolite synthesis, followed by a slower linear (steady-state) phase (Fig. 4C). This burst phenomenon has been observed in reactions in which the enzyme and substrate are components of the same membrane and has been speculated to be attributable to transient rapid metabolism of substrate in close proximity to the enzyme (35). Although analytical sensitivity constraints
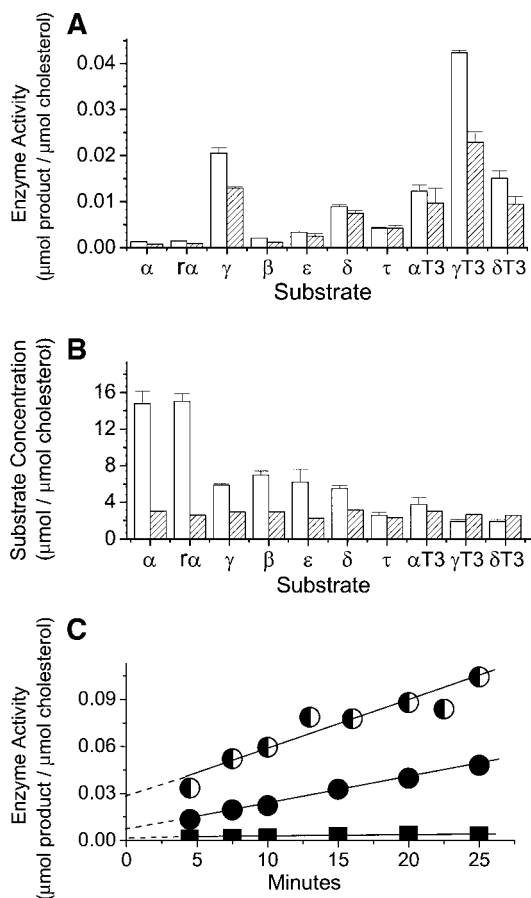
Fig. 4. A: Comparison of tocopherol-ω-hydroxylase activity of human liver microsomes incubated with various substrates under conditions of equimolar substrate concentration in the membrane. A total of 100 μg protein/ml human liver microsome was preincubated for 60 min with the concentrations of substrate-BSA complex predetermined to yield 100 nmol of substrate per milligram of protein in the microsomal membrane. A 20 min reaction was carried out either without (open bars) or after (striped bars) removal of the remaining aqueous (BSA-bound) substrate by 100,000 g centrifugation (see Materials and Methods). The reaction was initiated by the addition of 0.5 mM each NADPH and NAD. τ, tocol. B: Total amount of substrate in the reactions from A without (open bars) or after (striped bars) removal of the aqueous substrate pool. C: Time course of 13'-hydroxy-tocopherol + 13'-carboxy-tocopherol metabolite formation in human liver microsomes preincubated for 60 min with 25 μM substrate. The reaction was initiated by the addition of 1 mM each NADPH and NAD. The amount of burst-phase formation of metabolite is indicated by the y-intercept of the extrapolated (dashed) lines. Symbols and y-intercept (error): closed squares, α-TOH, 0.69×10^{-3} (0.34×10^{-3}); closed circles, γ-TOH, 6.34×10^{-3} (0.76×10^{-3}); half-closed/half-open circles, γ-T3, 29.0×10^{-3} (6.3×10^{-3}). Error bars represent means ± SD.

precluded the characterization of reaction kinetics within 4 min, the comparative magnitude of the burst, as reflected by y-intercept values, generally reflected the substrate activity pattern obtained with the longer reaction times. Together, the results in Fig. 4 show that tocopherol-ω-hydroxylase acts on the membrane-associated substrate pool. The overall rate of reaction may be influenced by both the efficiency of incorporation of substrates into the

bulk membrane and the rate at which they partition into the binding pocket of the enzyme.

Kinetics of vitamer metabolism

Kinetic analyses of microsomal tocochromanol metabolism were carried out during the linear steady-state phase with each individual substrate. Although the majority of tocopherols tested displayed typical Michaelis-Menten kinetics, the tocotrienols, and to a lesser extent δ-TOH and tocol, demonstrated concentration-dependent autoinhibition of their own metabolism at high substrate concentrations, as illustrated in **Fig. 5A** for γ-T3. Kinetic analyses of data from both human liver and CYP4F2 microsomes were carried out over a range of concentrations for the vitamers below those resulting in autoinhibition (Fig. 5A, B). RRR-α-TOH, rac-α-TOH, and rac-β-TOH displayed simple hyperbolic kinetics, whereas the other vitamers displayed sigmoidal kinetics indicative of allosteric enzymes. As such, kinetic constants were determined using OriginLab software to fit the data to both Michaelis-Menten (hyperbolic) and Hill (sigmoidal) curves, as shown below:

$$\text{Michaelis-Menten: V} = V_{max}[\text{S}]/(\text{km} + [\text{S}])$$

$$\text{Hill: V} = V_{max}[\text{S}]^n/(\text{k}^n + [\text{S}]^n)$$

The Hill equation differs from the Michaelis-Menten equation only in the variable n, the Hill coefficient, a measure of the positive (n > 1) or negative (n < 1) cooperativity of the enzyme with its substrate. A value of n = 1 indicates no cooperativity. Tocopherol-ω-hydroxylase substrates could be categorized into three groups: those fitting only Michaelis-Menten kinetics (α- and rac-α-TOH), those fitting both Michaelis-Menten and Hill kinetics (γ-, rac-γ-, β-, ε-, δ-TOH, and tocol), and those fitting Hill kinetics only (all three tocotrienols). The results indicate positive cooperativity for all three tocotrienols and either some or no cooperativity for the tocopherols. Determination of V_{max} and apparent K_m demonstrated a remarkably large variation in enzyme activity for the vitamers based on the aforementioned structural features, disentangled from the confounding effects of cell or membrane substrate incorporation. The pattern of substrate structural features on enzyme activity was similar between human liver microsomes and CYP4F2 microsomes. Apparent K_m values increased with the number of methyl groups, with α-TOH showing the least apparent affinity for the enzyme (or enzyme membrane system), compared with nonmethylated tocol and monomethylated δ-TOH, which exhibited the highest apparent affinities. There were no significant effects of methyl position (γ- vs. β- vs. ε-TOH) or side chain stereochemistry (RRR- vs. racemic α- or γ-TOH) on K_m. Unsaturation of the phytyl tail proved to exert the strongest effect on K_m, with all three tocotrienols displaying lower K_m values compared with their tocopherol counterparts.

V_{max}, a measure of maximal specific activity of the enzyme toward a substrate, was not directly related to the number of chromanol methyl groups, as the dimethyl γ-TOH exhibited the highest activity among the tocopherols. The position of the methyl group played a greater role,
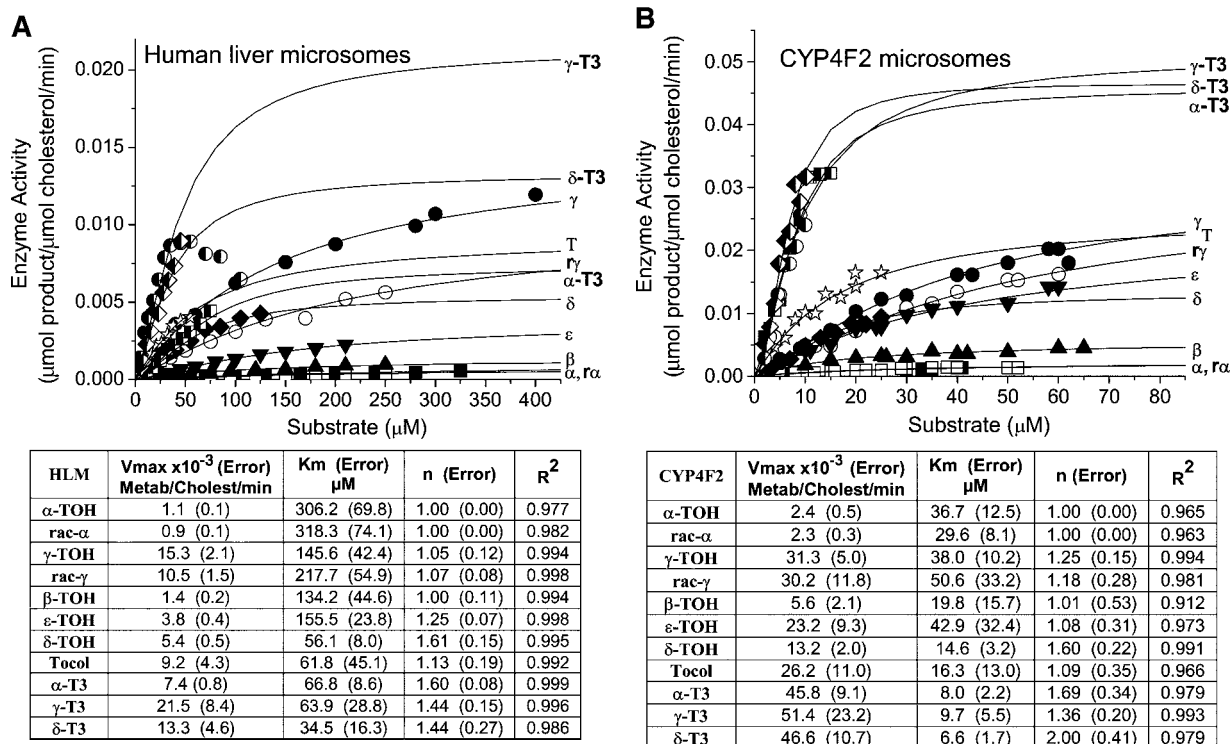
A Human liver microsomes — Enzyme Activity (μmol product/μmol cholesterol/min) vs Substrate (μM); curves labeled γ-T3, δ-T3, γ, T, rγ, α-T3, δ, ε, β, α, rα

B CYP4F2 microsomes — Enzyme Activity (μmol product/μmol cholesterol/min) vs Substrate (μM); curves labeled γ-T3, δ-T3, α-T3, γ, T, rγ, ε, δ, β, α, rα

HLM	Vmax x10⁻³ (Error) Metab/Cholest/min	Km (Error) μM	n (Error)	R²
α-TOH	1.1 (0.1)	306.2 (69.8)	1.00 (0.00)	0.977
rac-α	0.9 (0.1)	318.3 (74.1)	1.00 (0.00)	0.982
γ-TOH	15.3 (2.1)	145.6 (42.4)	1.05 (0.12)	0.994
rac-γ	10.5 (1.5)	217.7 (54.9)	1.07 (0.08)	0.998
β-TOH	1.4 (0.2)	134.2 (44.6)	1.00 (0.11)	0.994
ε-TOH	3.8 (0.4)	155.5 (23.8)	1.25 (0.07)	0.998
δ-TOH	5.4 (0.5)	56.1 (8.0)	1.61 (0.15)	0.995
Tocol	9.2 (4.3)	61.8 (45.1)	1.13 (0.19)	0.992
α-T3	7.4 (0.8)	66.8 (8.6)	1.60 (0.08)	0.999
γ-T3	21.5 (8.4)	63.9 (28.8)	1.44 (0.15)	0.996
δ-T3	13.3 (4.6)	34.5 (16.3)	1.44 (0.27)	0.986

CYP4F2	Vmax x10⁻³ (Error) Metab/Cholest/min	Km (Error) μM	n (Error)	R²
α-TOH	2.4 (0.5)	36.7 (12.5)	1.00 (0.00)	0.965
rac-α	2.3 (0.3)	29.6 (8.1)	1.00 (0.00)	0.963
γ-TOH	31.3 (5.0)	38.0 (10.2)	1.25 (0.15)	0.994
rac-γ	30.2 (11.8)	50.6 (33.2)	1.18 (0.28)	0.981
β-TOH	5.6 (2.1)	19.8 (15.7)	1.01 (0.53)	0.912
ε-TOH	23.2 (9.3)	42.9 (32.4)	1.08 (0.31)	0.973
δ-TOH	13.2 (2.0)	14.6 (3.2)	1.60 (0.22)	0.991
Tocol	26.2 (11.0)	16.3 (13.0)	1.09 (0.35)	0.966
α-T3	45.8 (9.1)	8.0 (2.2)	1.69 (0.34)	0.979
γ-T3	51.4 (23.2)	9.7 (5.5)	1.36 (0.20)	0.993
δ-T3	46.6 (10.7)	6.6 (1.7)	2.00 (0.41)	0.979

Fig. 5. Kinetic analysis of tocopherol-ω-hydroxylase substrate metabolism in human liver microsomes (HLM) and insect cell microsomes selectively expressing human CYP4F2. Kinetic parameters for human liver microsome or CYP4F2 microsome are listed below the corresponding graph. Kinetic fit to Michaelis-Menten or Hill equation was done using OriginLab 7.0 curve-fitting software as described in Materials and Methods. A total of 100 μg protein/ml human liver microsome (A) or 20 pmol cytochrome P450/ml CYP4F2 microsome (B) was preincubated with vitamer-BSA complex for 60 min. Ten or 20 min reaction was initiated by the addition of 1 mM each NADPH and NAD. Closed squares, α-TOH; open squares, rac-α-TOH; closed circles, γ-TOH; open circles, rac-γ-TOH; closed triangles, β-TOH; inverted closed triangles, ε-TOH; closed diamonds, δ-TOH; open stars, tocol; half-closed/half-open squares, α-T3; half-closed/half-open circles, γ-T3; half-closed/half-open diamonds, δ-T3.

with the C-5 methyl group greatly attenuating activity, as shown by the low activities of α- and β-TOH. The presence of a C-7 methyl group had the greatest positive effect, with greater activities toward γ- and ε-TOH. The lower observed activity toward ε-TOH compared with γ-TOH may be attributed to the inhibitory 5-C methyl group in ε-TOH. The stereochemistry of the side chain had no effect on the specific activity of the enzyme. Tocotrienols displayed high V_{max} values compared with the analogous tocopherols, again illustrating the strong influence of side chain unsaturation.

α-TOH-mediated stimulation of vitamer metabolism

The effect of the vitamers on the metabolism of each other was investigated in rat liver microsomes. The rate of ω-oxidation of γ-TOH was determined in the presence of several concentrations of RRR-α-, rac-α-, β-, or δ-TOH and γ-T3 (**Fig. 6**). γ-T3, itself an excellent substrate, inhibited γ-TOH metabolism even at low concentrations. δ-TOH, itself metabolized at rates more comparable to γ-TOH, was a more moderate inhibitor of γ-TOH metabolism. In contrast, no inhibition was observed by RRR-α-, rac-α, and β-TOHs. Rather, these substrates were found to increase the metabolism of γ-TOH, with the greatest stimulation by the α vitamers regardless of side chain stereochemistry. Sub-

sequent experiments revealed a stimulatory effect of α-TOH toward all tocopherols and tocotrienols tested (data not shown). The stimulatory effect was observed in both human liver and CYP4F2 microsomes and did not involve an effect on membrane substrate incorporation. At high concentrations of α-TOH (500 μM), the stimulation disappeared and α-TOH became inhibitory.

We suspected that α-TOH influenced the apparent allosteric interaction between tocopherol-ω-hydroxylase and its substrates. Kinetic analyses of the metabolism of γ-TOH or d₆-α-TOH were carried out in the presence or absence of 50 μM α-TOH (**Fig. 7**). In the absence of α-TOH, γ-TOH displayed sigmoidal kinetics and nonlinear Lineweaver-Burk kinetics typical of homotropic cooperativity. In the presence of α-TOH, the kinetics became hyperbolic and the Lineweaver-Burk analysis reverted to linearity (data not shown). A similar phenomenon has been reported for cholesterol and 7-ketocholesterol metabolism by the membrane-bound acyl-CoA:cholesterol acyltransferase (36). Additionally, the V_{max} of the reaction increased in the presence of α-TOH, whereas the apparent K_m was unchanged. To test whether α-TOH stimulated its own metabolism, RRR-d₆-α-TOH was used as a substrate to distinguish it from the unlabeled α-TOH. Unlike its effects on the non-α vitamers, α-TOH did not influence its own metabolism.
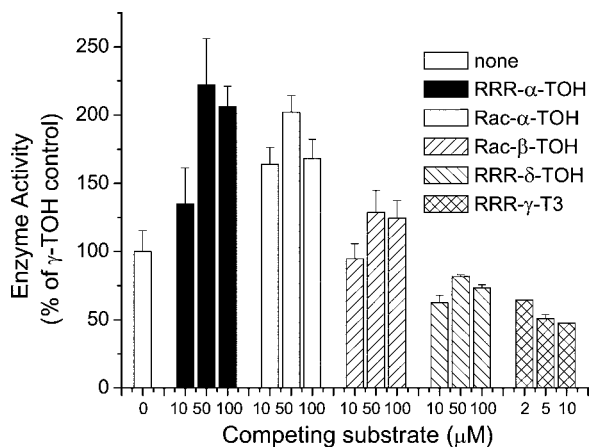
Fig. 6. Influence of various tocopherol-ω-hydroxylase substrates on the metabolism of γ-TOH in rat liver microsomes. A total of 100 μg/ml microsomal protein was preincubated with 10 μM γ-TOH-BSA complex with or without the addition of several concentrations of various competing substrates. A 20 min reaction was then initiated by the addition of 0.5 mM each NADPH and NAD, and the combined levels of the 13′-hydroxy-tocopherol + 13′-carboxy-tocopherol metabolites generated was measured per microsomal cholesterol. Data are expressed as a percentage of the γ-TOH-only (control) reaction. Error bars represent means ± SD.

DISCUSSION

Despite apparent similar absorption efficiencies of the various forms of vitamin E (37), mammals retain predominantly α-TOH and only small quantities of other forms. We previously identified the first enzyme catalyzing the biotransformation of vitamin E and demonstrated its preferential metabolism of γ-TOH over α-TOH (30). This enzyme, CYP4F2, is expressed in human liver, kidney, and intestinal cells (38) and is constitutively expressed in hepatoblastoma HepG2 and lung adenocarcinoma A549 cell lines (39). Here, we systematically characterized the enzyme activity with respect to key structural features of its tocochromanol substrates.

In HepG2 cultures, human liver microsomes, and CYP4F2 microsomes, the two substrate structural features most determinant of tocopherol-ω-hydroxylase activity were the presence of double bonds in the side chain and the positions of the methyl groups around the chromanol ring. The degree to which α-T3 was metabolized compared with α-TOH indicates the dominance of side chain unsaturation over the inhibitory effect of the methyl group at carbon 5 of the chromanol ring. Although the number of methyl groups initially appeared to play a role in the differences in activity, these differences most likely indirectly reflected the effects of methyl position. Racemerization about the three chiral carbons of the side chain did not significantly affect the rate of metabolism of α-TOH, indicating that the preferential retention of the RRR form of α-TOH in vivo is probably entirely attributable to its higher affinity for α-TTP.

The possibility that other P450 enzymes that metabolize vitamin E exist in the liver seems unlikely based on the evidence presented here. The kinetics of metabolism in
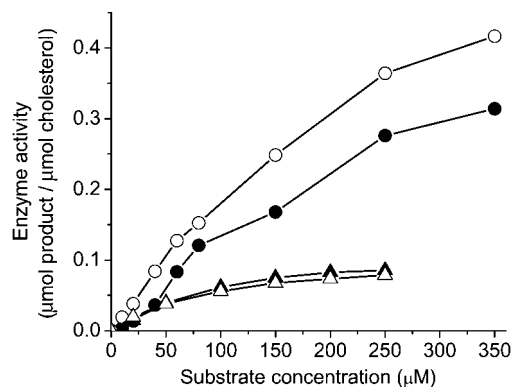
	Vmax (error)	Kmapp (error)
γ-TOH	0.471 (0.106)	205.1 (77.3)
γ-TOH + α-TOH	0.722 (0.086)*	258.0 (57.1)
d6α-TOH	0.101 (0.002)	70.3 (3.0)
d6α-TOH + α-TOH	0.109 (0.003)	93.4 (5.2)

Fig. 7. Influence of α-TOH on the metabolism of γ-TOH or d_6-α-TOH in rat liver microsomes. A total of 100 μg/ml microsomal protein was preincubated for 60 min with substrate in the presence or absence of 50 μM α-TOH, followed by a 20 min reaction initiated by the addition of 0.5 mM each NADPH and NAD. Kinetic parameters are given (±SEM) based on best fit to the Hill equation. Significant difference in kinetic parameters with or without 50 μM α-TOH is indicated by the asterisk ($P < 0.05$). Closed circles, γ-TOH; open circles, γ-TOH + 50 μM α-TOH; closed triangles, d_6-α-TOH; open triangles, d_6-α-TOH + 50 μM α-TOH.

human liver microsomes was similar to that of microsomes containing only CYP4F2, with no evidence of the biphasic kinetics normally associated with two enzymes acting on the same substrate. Additionally, the strikingly similar pattern of the complete ω/β-oxidation represented by the hepatocyte system and the microsomal systems expressing only the ω-hydroxylation activity strongly suggests that the tocopherol-ω-hydroxylase-mediated hydroxylation reaction is the only discriminatory step of the entire side chain truncation pathway.

In whole cells, tocochromanol substrates not only accumulated at varying rates, but access to the inner organelle membranes also varied greatly, likely resulting in different concentrations in the vicinity of the enzyme. Similarly, analysis of microsomes incubated with BSA-complexed substrates revealed large differences in uptake, with a pattern that generally paralleled that seen in whole cells. As such, the observed kinetics of metabolism by the membrane-associated tocopherol-ω-hydroxylase may be influenced by factors not relevant to aqueous enzymes (35). Differences in the rates of membrane association of the substrates as well as differences in the rates of lateral diffusion through the membrane may influence the apparent affinity of the enzyme for different vitamers. Therefore, the apparent K_m is probably best interpreted as reflecting the affinity of substrate for the enzyme-membrane complex. V_{max}, which is not influenced by substrate access to the enzyme, is likely a more useful indicator of the substrate specificity of CYP4F2 per se. Fluorescence quenching data suggest that tocopherols with fewer methyl groups localize progressively deeper in the membrane bilayer (40). Differential

localization may result in differences in membrane uptake or mobility and access of the tocochromanol substrates to the ligand binding domain(s) of CYP4F2, whose tertiary structure has not been modeled.

The positive cooperativity exhibited by CYP4F2 toward certain tocochromanols was unexpected, as evidence of allostery has not been reported for its previously characterized substrates (32, 41, 42). The underlying mechanism is unclear at present and may involve either direct substrate-enzyme interaction or modulation of the membrane environment. CYP3A4 displays both homotropic and heterotropic cooperativity, attributed variously to the binding of multiple substrate molecules within a single binding site or at distinct sites (43–46). Although in this case direct interaction seems supported by the specificity of the effect (no cooperativity with α- or β-TOHs), a membrane-mediated effector mechanism of substrate or products (the latter of which likely remain in the membrane) cannot be ruled out (47). The positive effector feature of α-TOH, however, is probably not attributable to a general effect on membrane fluidity. α-TOH reduces membrane fluidity, as determined by several biophysical techniques (6, 48, 49). A reduction in membrane fluidity induced by cholesterol enrichment or phospholipid modification inhibits the activity of CYP7A and CYP3A (50). Conversely, an increase in fluidity induced by n-octanol enhances the activity of membrane sialidase and reduces K_m with no effect on V_{max} (51). In contrast, α-TOH increases the V_{max} of the metabolism of γ-TOH with no effect on K_m. However, regardless of mechanism, the stimulation of the catabolism of γ-TOH by α-TOH reported here may contribute to the in vivo suppression of serum γ-TOH and the increased excretion of γ-CEHC upon supplementation with α-TOH (33, 52).

The effects of substrate structure on tocopherol-ω-hydroxylase activity are informative in light of the relative affinities of the various tocochromanols for the hepatic α-TOH transfer protein, as reported by Panagabko et al. (21). These affinities are for the most part the inverse of their ability to act as ω-hydroxylase substrates, suggesting a collaborative role for these two proteins in producing and maintaining the RRR-α-TOH-enriched phenotype. The fact that α-TTP shows a high degree of specificity for the RRR stereochemistry of the side chain, whereas tocopherol-ω-hydroxylase does not, suggests that α-TTP is the main regulatory entity underlying the preferential retention of natural over synthetic α-TOH. However, plasma of α-TTP knockout mice is still enriched in α-TOH relative to γ-TOH, although total tocopherol levels are greatly reduced relative to the wild type (53). Together, these data support the concept that in mammals α-TTP is predominantly a vitamin E retention factor, whereas selectivity among the different vitamers is primarily driven by the substrate specificity of tocopherol-ω-hydroxylase. The fact that *Drosophila* exhibits tocopherol selectivity via a cytochrome P450 but apparently lacks α-TTP suggests a separate evolution of these mechanisms and a fundamental selective advantage of the trait of selectivity (54).

In summary, the substrate specificity of tocopherol-ω-hydroxylase, an activity of the human enzyme CYP4F2, was systematically characterized. The positions of methyl groups about the chromanol ring, particularly at carbon 5, and unsaturation of the side chain were critical determinants of the rate of ω-hydroxylation. The inverse relationship between the rate of metabolism of the various forms of vitamin E and their bioavailability in vivo illustrates the centrality of this metabolic pathway in determining the biopotency of the tocochromanols. Elucidation of the mechanisms regulating vitamin E status, and in particular the molecular basis underlying the discriminatory elimination of certain forms of vitamin E, may facilitate strategies for manipulating the levels of such forms for specific therapeutic purposes.▨

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