Affinity for α-tocopherol transfer protein as a determinant of the biological activities of vitamin E analogs

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Abstract **α-Tocopherol transfer protein (αTTP), a product of the gene which causes familial isolated vitamin E deficiency, plays an important role in determining the plasma vitamin E level. We examined the structural characteristics of vitamin E analogs required for recognition by αTTP. Ligand specificity was assessed by evaluating the competition of non-labeled vitamin E analogs and α-[^{3}H]tocopherol for transfer between membranes in vitro. Relative affinities (*RRR*-α-tocopherol = 100%) calculated from the degree of competition were as follows: β-tocopherol, 38%; γ-tocopherol, 9%; δ-tocopherol, 2%; α-tocopherol acetate, 2%; α-tocopherol quinone, 2%; *SRR*-α-tocopherol, 11%; α-tocotrienol, 12%; trolox, 9%. Interestingly, there was a linear relationship between the relative affinity and the known biological activity obtained from the rat resorption-gestation assay. From these observations, we conclude that the affinity of vitamin E analogs for αTTP is one of the critical determinants of their biological activity.**

Key words: Vitamin E; α-Tocopherol; Transfer protein; Ligand specificity; Anti-oxidant

1. Introduction

Vitamin E is a fat-soluble antioxidant that prevents lipid oxidation in biological membranes[1]. Vitamin E occurs in nature in eight different forms: α-, β-, γ- and δ-tocopherols (which have a chromanol ring and phytyl tail and differ in the number and position of methyl groups on the ring) and α-, β-, γ- and δ-tocotrienols (which have unsaturated tails). Synthetic α-tocopherol, sold as a vitamin E supplement, contains stereoisomers arising from different configurations of the phytyl tail. It has long been recognized that the antioxidant activities of the various forms of vitamin E are usually unrelated to their biological activities [2,3].

α-Tocopherol transfer protein (αTTP), which binds this vitamin and enhances its transfer between membranes, is present in the liver cytosol of animals, including rats [4] and humans [5]. Recently, we have cloned the gene encoding αTTP from rats and humans and demonstrated it to be the causative gene for familial isolated vitamin E deficiency [6,7]. Patients affected by this disease have remarkably low plasma levels of α-tocopherol [8,9]. These findings established liver αTTP as a critical factor in determining the plasma α-tocopherol level. Previously, we showed that αTTP binds to α-tocopherol in preference to other tocopherol analogs [4,10]. However, detailed studies have not fully classified the structural requirements needed for recognition by αTTP. In the present investigation, we analyzed ligand specificity for αTTP in more detail and demonstrated that the relative affinity of tocopherol analogs for αTTP correlates well with their biological activity.

2. Materials and methods

2.1. Materials

Egg-yolk phosphatidylcholine was prepared by chromatography on neutral aluminium oxide and silica acid. D-α-[^{3}H]tocopherol acetate (11.3 Ci/mmol) was purchased from Amersham (Buckinghamshire, UK). Glycerol tri[carboxyl-^{14}C]oleate (112 mCi/mmol) was purchased from NEN (Dreieich, Germany). *RRR*-α-, β-, γ- and δ-tocopherol, α-tocotrienol, *RRR*-α-tocopherol acetate and 2-*ambo*-α-tocopherol acetate were kindly donated by Eisai Co. (Tokyo, Japan). *SRR*-α-tocopherol was purified from 2-*ambo*-α-tocopherol acetate as described previously [11].

2.1.1. Purification of D-α-[^{3}H]tocopherol from D-α-[^{3}H]tocopherol acetate. The procedure was based on the experiments of Syvaoja and Salminen [12]. D-α-[^{3}H]Tocopherol acetate was saponified and the resulting *d*-α-[^{3}H]tocopherol was extracted with hexane and purified by silica gel G thin layer chromatography.

2.2. Preparation of donor liposomes

Liposomes composed of egg-yolk phosphatidylcholine, dicetylphosphate and butylhydroxytoluene (molar ratio 10:1:0.5)with traces of α-[^{3}H]tocopherol (4.0×10^6 dpm) and glycerol tri[^{14}C]oleate (5.5×10^5 dpm) as non-exchangeable markers were prepared as described previously [10].

2.3. Preparation of an acceptor membrane fraction from rat liver

A crude membrane fraction was prepared from male Sprague-Dawley rat (350–500 g) livers according to a previously described procedure [4].

2.4. Determination of the transfer of α-tocopherol from the liposomes to the crude membrane fraction

The procedure was based on the experiments of Bloj and Zilversmit [13]. A given amount of liposomes (0.07 μmol phospholipid/tube) was incubated with the membranes (0.05 mg protein) in the presence or absence of purified αTTP in 1 ml of buffer A (0.25 M sucrose, 1 mM EDTA, 10 mM Tris-HCl, pH 7.4). After incubation at 37°C for 30 min, the membranes were precipitated by centrifugation at $15000\times g$ for 15 min, and the radioactivity in 0.8 ml of supernatant was counted. Using this procedure, approximately 90% of the liposomes were recovered in the supernatant. The transfer of α-[^{3}H]tocopherol from the liposomes to mitochondria was calculated from the following equation:

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Abbreviations: αTTP, α-tocopherol transfer protein

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$(1-{}^3H/{}^{14}C$ content of liposomes after incubation$/{}^3H/{}^{14}C$ content of liposomes before incubation$)\times 100\%$ (1)

2.5. Purification of αTTP

αTTP was purified from male Sprague-Dawley rat (350–500 g) livers as described previously [4]. The purified protein was frozen in liquid nitrogen and stored at −80°C until use. Protein concentrations were determined by the Lowry method [14].

2.6. Determination of the apparent IC_{50} of various tocopherol analogs

K_M and V_{max} were estimated according to the following equation:

$$v_c = V_{max}\times[S]/K_M + [S] \quad (2)$$

in which v_c is the apparent transfer rate of α-[^{3}H]tocopherol in the absence of another tocopherol analog, [S] is the concentration of α-[^{3}H]tocopherol, V_{max} is the maximum transfer rate of α-[^{3}H]tocopherol and K_M is the Michaelis constant. Since [S] is much smaller than K_M, Eq. 2 becomes:

$$v_c = V_{max}\times[S]/K_M \quad (3)$$

Solving for V_{max}/K_M in Eq. 3:

$$V_{max}/K_M = v_c/[S] \quad (4)$$

As the inhibition of α-[^{3}H]tocopherol transport by the various tocopherol analogs was competitive, the apparent IC_{50} value was determined from the following Michaelis-Menten equation:

$$v_i = V_{max}\times[S]/K_M(1 + [I]/IC_{50}) + [S] \quad (5)$$

in which v_i is the concentration of α-[^{3}H]tocopherol and [I] is the concentration of the other tocopherol analogs. Since [S] is much smaller than K_M, Eq. 5 becomes:

$$v_i = V_{max}\times[S]/K_M(1 + [I]/IC_{50}) \quad (6)$$

Substituting for V_{max}/K_M in Eq. 6:

$$v_i = v_c/1 + [I]/IC_{50} \quad (7)$$

Solving for IC_{50} in Eq. 7:

$$IC_{50} = [I]/(v_c/v_i - 1) \quad (8)$$

The relative affinity was then calculated using the following equation:

$$\text{Relative affinity} = 1/IC_{50} \quad (9)$$

3. Results and discussion

Under the conditions used for the determination of αTTP activity, the reaction was linearly dependent on the purified αTTP protein concentration up to 40 ng (Fig. 1). The reaction proceeded linearly for up to 30 min at 37°C (data not shown). A variety of vitamin E analogs were tested for their ability to compete for transfer between membranes. First, liposomes containing varying amounts of non-labeled α-tocopherol were subjected to the αTTP assay. As shown in Fig. 2, the transfer of radioactive α-tocopherol was progressively reduced by increasing concentrations of non-labeled α-tocopherol, in-

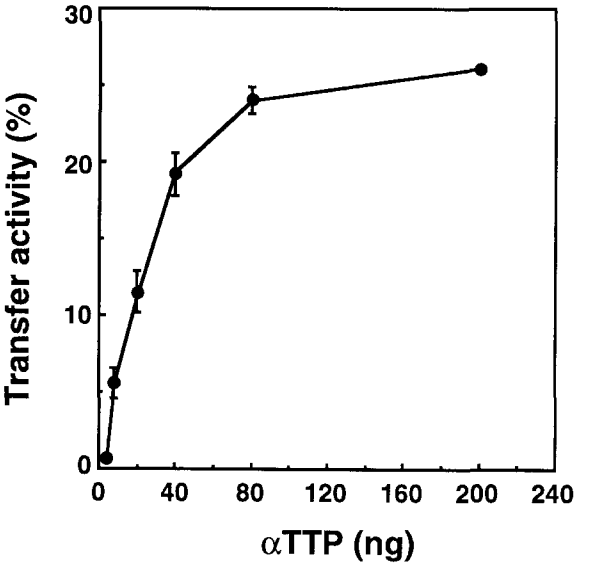

Fig. 1. Dose-response relationship of αTTP and α-tocopherol transfer activity. Liposomes were incubated with membranes at 37°C for 30 min in the presence of various amounts of purified αTTP isoform (I). Each point denotes the mean ± SE of three experiments. The membranes were then precipitated by centrifugation and the radioactivity of aliquots of supernatant was measured. The percentage of α-tocopherol transferred was calculated as described in Section 2.

Table 1
Relative affinities of various tocopherol analogs for αTTP isoform (I)

Competitors	Relative affinity(%)
α-Tocopherol	100
β-Tocopherol	38.1 ± 9.3
γ-Tocopherol	8.9 ± 0.6
δ-Tocopherol	1.6 ± 0.3
α-Tocopherol acetate	1.7 ± 0.1
α-Tocopherol quinone	1.5 ± 0.1
SRR-α-Tocopherol	10.5 ± 0.4
α-Tocotrienol	12.4 ± 2.3
Trolox	9.1 ± 1.2

Relative affinities of various tocopherol analogs were calculated from Eq. 9 as described in Section 2, taking the relative affinity of α-tocopherol as 100. Each point denotes the mean ± SE of three experiments.

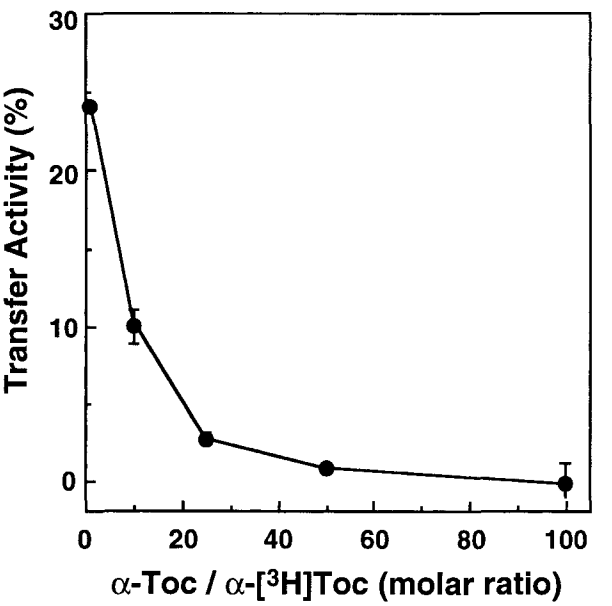

Fig. 2. Inhibition of αTTP isoform (I)-mediated α-[^{3}H]tocopherol transfer by various amounts of unlabeled α-tocopherol. Liposomes composed of egg yolk phosphatidylcholine, bis(hexadecanyl)-phosphate and various amounts of unlabeled α-tocopherol with traces of radioactive α-tocopherol (5.7×10^4 dpm) were incubated with mitochondria at 37°C for 30 min in the presence of 40 ng purified αTTP. Each point denotes the mean ± SE of three experiments.

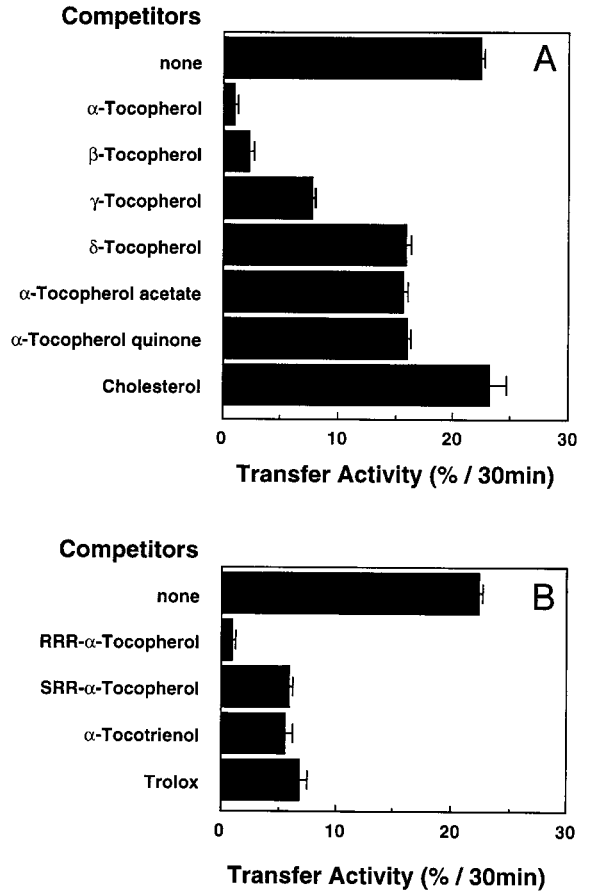

Fig. 3. Effect of the incorporation of unlabeled tocopherol analogs into liposomes on the transfer of α-[^{3}H]tocopherol. Liposomes composed of egg yolk phosphatidylcholine, bis(hexadecanyl)-phosphate and chromanol (A) or side chain (B) analogs of α-tocopherol (molar ratio 10:1:0.16, 0.07 μmol phospholipid/tube) with traces of radioactive α-tocopherol (5.7×10^4 dpm) were incubated with membranes at 37°C for 30 min in the presence of 40 ng purified αTTP. Each point denotes the means ± SE of three experiments.

dicating that the amount of radioactive tocopherol, which acted as a substrate for αTTP, in the donor liposomes was saturated under the present assay conditions.

Initial studies examined the inhibitory effects of vitamin E analogs with differing numbers of methyl groups on their chromanol rings or those without a free hydroxyl group on the transfer of radioactive α-tocopherol. For this, a 50-fold excess of each analog was added to the donor liposomes in addition to radioactive α-tocopherol, and the effect on the transfer of radioactive α-tocopherol was examined. Under these conditions, α-, β-, γ- and δ-tocopherols inhibited the activity by 0.9%, 2.2%, 6.7%, 15.8%, respectively (Fig. 3). The following values were obtained from Eq. 9 (with *RRR*-α-tocopherol = 100%): β-tocopherol, 38.1%; γ-tocopherol, 8.9%; δ-tocopherol, 1.6% (Table 1). These data suggest that all three methyl groups are important for recognition by αTTP, but that the methyl group at position 5 on the chromanol ring is especially important in the light of the difference in affinity between β- and γ-tocopherols. α-Tocopherol acetate and α-tocopheryl quinone, both of which have no free hydroxyl group, are poor substrates for αTTP (relative affinities are about 2%).

The influence of the side-chain on the transfer was analyzed by a second series of investigations. For this, we used *SRR*-α-tocopherol, a stereoisomer of *RRR*-α-tocopherol, α-tocotrienol, which has an unsaturated tail, and trolox, which is α-tocopherol with a carboxyl group instead of the phytyl tail. It was found that these analogs had approximately the same potency to inhibit αTTP, the relative affinity for αTTP being calculated as approximately 10% (Table 1). These data indicate that although αTTP also recognizes the phytyl chain structure and its orientation, a tocopherol analog without a side chain still possesses 10% affinity for αTTP compared with one containing the phytyl chain.

Considerable efforts have been made to determine the biological activity of the various forms of vitamin E [3,15–17]. The biological activity of α-tocopherol and its analogs has been determined using various physiological, biochemical and chemical tests. Among the functional tests, the rat resorption-gestation test and the in vivo hemolysis test have been widely accepted. Here, the known biological activity of each analogs obtained using the resorption-gestation test [18] was plotted against the relative affinity for αTTP. Interestingly, as shown in Fig. 4, there was a good linear relationship between relative affinity and biological activity.

It has long been recognized that the antioxidant activities of the various forms of vitamin E are not consistent with their biological activities. From these observations, it has been hypothesized α-tocopherol may have a specific function other than antioxidant activity. In fact, α-tocopherol is known to be a potent inhibitor of protein kinase C [19] or phospholipase A2 [20], although its physiological significance is not yet clear. Catignani and Bieri first noted that the biological activities of the various forms of vitamin E were similar to their ability to compete for binding to the tocopherol binding protein [21]. The present data strongly support this notion.

The function of αTTP is to incorporate vitamin E taken up by liver cells into very low-density lipoproteins. The tissues become enriched with vitamin E by a variety of non-specific

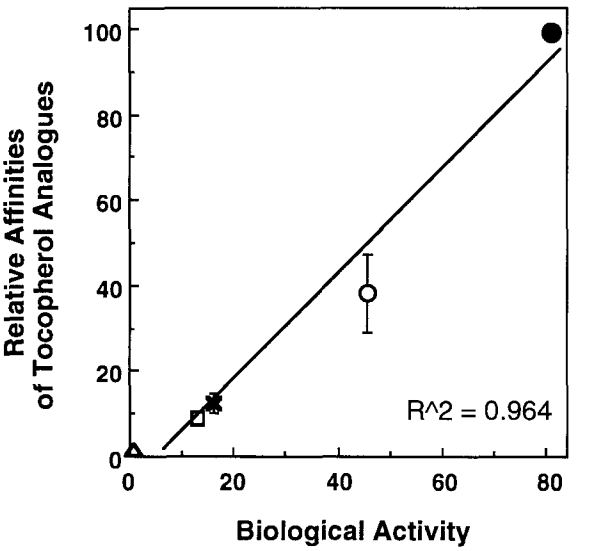

Fig. 4. Correlation of the biological activities of tocopherol analogs with their affinities for αTTP. Relative affinities of β- (○), γ- (□) and δ-tocopherol (△) and α-tocotrienol (×) were calculated from Eq. 9 as described in Section 2, taking the relative affinity of α-tocopherol (●) as 100. Each point denotes the mean ± SE of three experiments. Biological activities are those determined by Leth and Sondergaard [18].

mechanisms that depend upon the normal metabolism of lipoproteins. The biological activity of vitamin E is thus dependent upon its delivery to tissues, and reductions in the binding capacity or affinity of αTTP will limit the secretion of the various forms of vitamin E into lipoproteins and the subsequent delivery of vitamin E by these lipoproteins to tissues. The biological activity of various vitamin E analogs may be determined by a number of factors including their chemical antioxidant activities and physicochemical natures. From the result of this study, we propose that the affinity of vitamin E analogs for αTTP, which may in turn determine their plasma levels, is one of the major determinants of their biological activity.

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