

# Affinity for $\alpha$ -tocopherol transfer protein as a determinant of the biological activities of vitamin E analogs

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**Abstract**  $\alpha$ -Tocopherol transfer protein ( $\alpha$ TTP), 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  $\alpha$ TTP. Ligand specificity was assessed by evaluating the competition of non-labeled vitamin E analogs and  $\alpha$ -[<sup>3</sup>H]tocopherol for transfer between membranes in vitro. Relative affinities (*RRR*- $\alpha$ -tocopherol=100%) calculated from the degree of competition were as follows:  $\beta$ -tocopherol, 38%;  $\gamma$ -tocopherol, 9%;  $\delta$ -tocopherol, 2%;  $\alpha$ -tocopherol acetate, 2%;  $\alpha$ -tocopherol quinone, 2%; *SRR*- $\alpha$ -tocopherol, 11%;  $\alpha$ -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  $\alpha$ TTP is one of the critical determinants of their biological activity.

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**Key words:** Vitamin E;  $\alpha$ -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:  $\alpha$ -,  $\beta$ -,  $\gamma$ - and  $\delta$ -tocopherols (which have a chromanol ring and phytyl tail and differ in the number and position of methyl groups on the ring) and  $\alpha$ -,  $\beta$ -,  $\gamma$ - and  $\delta$ -tocotrienols (which have unsaturated tails). Synthetic  $\alpha$ -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].

$\alpha$ -Tocopherol transfer protein ( $\alpha$ TTP), 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  $\alpha$ TTP 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  $\alpha$ -tocopherol [8,9]. These findings established liver

$\alpha$ TTP as a critical factor in determining the plasma  $\alpha$ -tocopherol level. Previously, we showed that  $\alpha$ TTP binds to  $\alpha$ -tocopherol in preference to other tocopherol analogs [4,10]. However, detailed studies have not fully classified the structural requirements needed for recognition by  $\alpha$ TTP. In the present investigation, we analyzed ligand specificity for  $\alpha$ TTP in more detail and demonstrated that the relative affinity of tocopherol analogs for  $\alpha$ TTP 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- $\alpha$ -[<sup>3</sup>H]tocopherol acetate (11.3 Ci/mmol) was purchased from Amersham (Buckinghamshire, UK). Glycerol tri[carboxyl-<sup>14</sup>C]oleate (112 mCi/mmol) was purchased from NEN (Dreieich, Germany). *RRR*- $\alpha$ -,  $\beta$ -,  $\gamma$ - and  $\delta$ -tocopherol,  $\alpha$ -tocotrienol, *RRR*- $\alpha$ -tocopherol acetate and 2-*ambo*- $\alpha$ -tocopherol acetate were kindly donated by Eisai Co. (Tokyo, Japan). *SRR*- $\alpha$ -tocopherol was purified from 2-*ambo*- $\alpha$ -tocopherol acetate as described previously [11].

**2.1.1. Purification of D- $\alpha$ -[<sup>3</sup>H]tocopherol from D- $\alpha$ -[<sup>3</sup>H]tocopherol acetate.** The procedure was based on the experiments of Syvaaja and Salminen [12]. D- $\alpha$ -[<sup>3</sup>H]Tocopherol acetate was saponified and the resulting D- $\alpha$ -[<sup>3</sup>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  $\alpha$ -[<sup>3</sup>H]tocopherol ( $4.0 \times 10^6$  dpm) and glycerol tri[<sup>14</sup>C]oleate ( $5.5 \times 10^5$  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 $\alpha$ -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  $\mu$ mol phospholipid/tube) was incubated with the membranes (0.05 mg protein) in the presence or absence of purified  $\alpha$ TTP 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  $\alpha$ -[<sup>3</sup>H]tocopherol from the liposomes to mitochondria was calculated from the following equation:

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**Abbreviations:**  $\alpha$ TTP,  $\alpha$ -tocopherol transfer protein

$$\frac{(\text{content of liposomes after incubation} / \text{content of liposomes before incubation}) \times 100\%}{\text{content of liposomes before incubation}} \times 100\% \quad (1)$$

### 2.5. Purification of $\alpha$ TTP

$\alpha$ TTP 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  $-80^{\circ}\text{C}$  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  $\alpha$ - $^3\text{H}$ tocopherol in the absence of another tocopherol analog,  $[S]$  is the concentration of  $\alpha$ - $^3\text{H}$ tocopherol,  $V_{max}$  is the maximum transfer rate of  $\alpha$ - $^3\text{H}$ 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  $\alpha$ - $^3\text{H}$ 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  $\alpha$ - $^3\text{H}$ 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  $\alpha$ TTP activity, the reaction was linearly dependent on the purified  $\alpha$ TTP protein concentration up to 40 ng (Fig. 1). The reaction

Table 1  
Relative affinities of various tocopherol analogs for  $\alpha$ TTP isoform (I)

Competitors	Relative affinity(%)
$\alpha$ -Tocopherol	100
$\beta$ -Tocopherol	$38.1 \pm 9.3$
$\gamma$ -Tocopherol	$8.9 \pm 0.6$
$\delta$ -Tocopherol	$1.6 \pm 0.3$
$\alpha$ -Tocopherol acetate	$1.7 \pm 0.1$
$\alpha$ -Tocopherol quinone	$1.5 \pm 0.1$
SRR- $\alpha$ -Tocopherol	$10.5 \pm 0.4$
$\alpha$ -Tocotrienol	$12.4 \pm 2.3$
Trolox	$9.1 \pm 1.2$

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

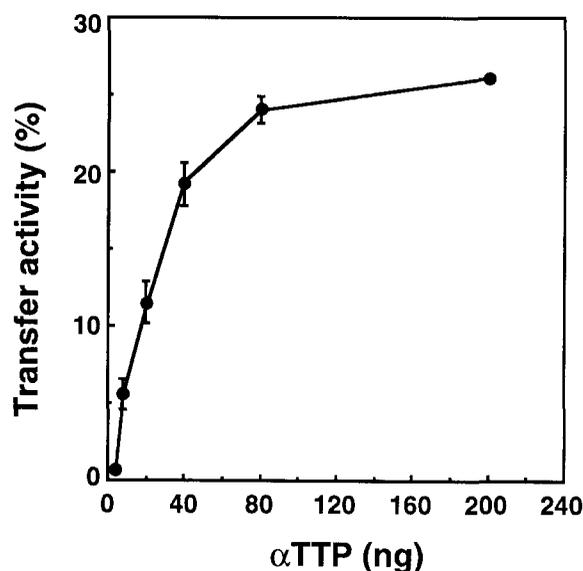


Fig. 1. Dose-response relationship of  $\alpha$ TTP and  $\alpha$ -tocopherol transfer activity. Liposomes were incubated with membranes at  $37^{\circ}\text{C}$  for 30 min in the presence of various amounts of purified  $\alpha$ TTP isoform (I). Each point denotes the mean  $\pm$  SE of three experiments.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

proceeded linearly for up to 30 min at  $37^{\circ}\text{C}$  (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  $\alpha$ -tocopherol were subjected to the  $\alpha$ TTP assay. As shown in Fig. 2, the transfer of radioactive  $\alpha$ -tocopherol was progressively reduced by increasing concentrations of non-labeled  $\alpha$ -tocopherol, in-

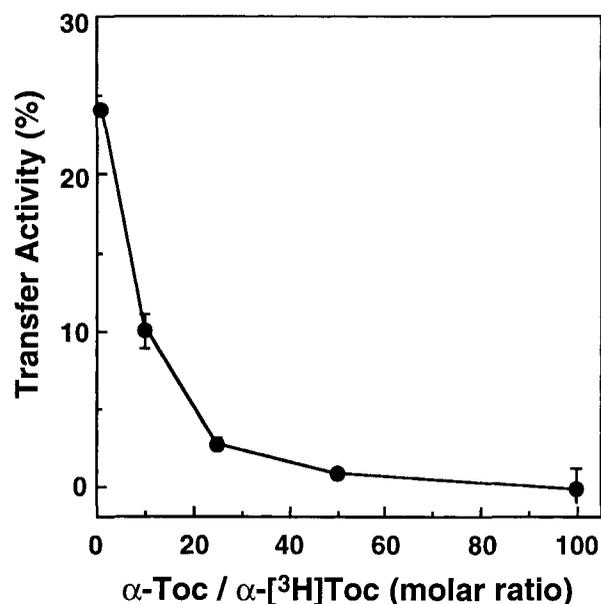


Fig. 2. Inhibition of  $\alpha$ TTP isoform (I)-mediated  $\alpha$ - $^3\text{H}$ tocopherol transfer by various amounts of unlabeled  $\alpha$ -tocopherol. Liposomes composed of egg yolk phosphatidylcholine, bis(hexadecanyl)-phosphate and various amounts of unlabeled  $\alpha$ -tocopherol with traces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

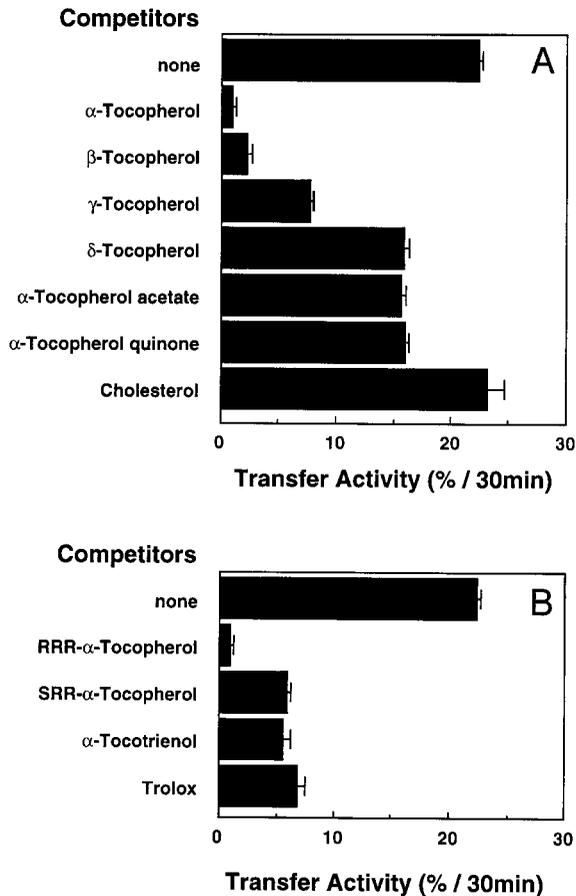


Fig. 3. Effect of the incorporation of unlabeled tocopherol analogs into liposomes on the transfer of  $\alpha$ -[ $^3$ H]tocopherol. Liposomes composed of egg yolk phosphatidylcholine, bis(hexadecanyl)-phosphate and chromanol (A) or side chain (B) analogs of  $\alpha$ -tocopherol (molar ratio 10:1:0.16,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

dicating that the amount of radioactive tocopherol, which acted as a substrate for  $\alpha$ TTP, 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  $\alpha$ -tocopherol. For this, a 50-fold excess of each analog was added to the donor liposomes in addition to radioactive  $\alpha$ -tocopherol, and the effect on the transfer of radioactive  $\alpha$ -tocopherol was examined. Under these conditions,  $\alpha$ -,  $\beta$ -,  $\gamma$ - and  $\delta$ -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- $\alpha$ -tocopherol = 100%):  $\beta$ -tocopherol, 38.1%;  $\gamma$ -tocopherol, 8.9%;  $\delta$ -tocopherol, 1.6% (Table 1). These data suggest that all three methyl groups are important for recognition by  $\alpha$ TTP, but that the methyl group at position 5 on the chromanol ring is especially important in the light of the difference in affinity between  $\beta$ - and  $\gamma$ -tocopherols.  $\alpha$ -Tocopherol acetate and  $\alpha$ -tocopheryl quinone, both of which have no free hydroxyl group, are poor substrates for  $\alpha$ TTP (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- $\alpha$ -tocopherol, a stereoisomer of RRR- $\alpha$ -tocopherol,  $\alpha$ -tocotrienol, which has an unsaturated tail, and trolox, which is  $\alpha$ -tocopherol with a carboxyl group instead of the phytyl tail. It was found that these analogs had approximately the same potency to inhibit  $\alpha$ TTP, the relative affinity for  $\alpha$ TTP being calculated as approximately 10% (Table 1). These data indicate that although  $\alpha$ TTP also recognizes the phytyl chain structure and its orientation, a tocopherol analog without a side chain still possesses 10% affinity for  $\alpha$ TTP 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  $\alpha$ -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  $\alpha$ TTP. 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  $\alpha$ -tocopherol may have a specific function other than antioxidant activity. In fact,  $\alpha$ -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  $\alpha$ TTP 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

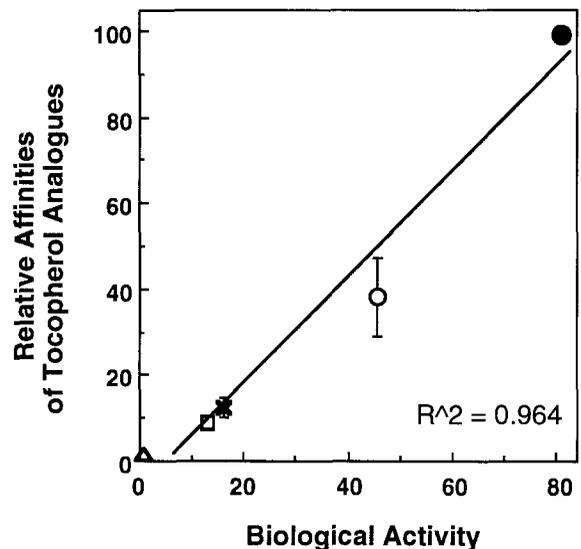


Fig. 4. Correlation of the biological activities of tocopherol analogs with their affinities for  $\alpha$ TTP. Relative affinities of  $\beta$ - (○),  $\gamma$ - (□) and  $\delta$ -tocopherol (△) and  $\alpha$ -tocotrienol (×) were calculated from Eq. 9 as described in Section 2, taking the relative affinity of  $\alpha$ -tocopherol (●) as 100. Each point denotes the mean  $\pm$  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  $\alpha$ TTP 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  $\alpha$ TTP, which may in turn determine their plasma levels, is one of the major determinants of their biological activity.

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