Note

A Kinetic Study of the Radical-Scavenging Action of Tocotrienols in the Membranes of Egg Yolk Phosphatidylcholine Vesicles

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Summary Vitamin E is localized in membranes and functions as an efficient inhibitor of lipid peroxidation in biological systems. In this study, we measured the second-order rate constants (k_s) for the reaction of tocotrienol homologues (α -, β -, γ -, and δ -Toc-3Hs) with the aroxyl radical ($\text{ArO}\cdot$) used as a model for lipid peroxyl radicals ($\text{LOO}\cdot$) in the membranes of egg yolk phosphatidylcholine (EYPC) vesicles by stopped-flow spectrophotometry, and compared them to those of tocopherol homologues (α -, β -, γ -, and δ -TocHs). The relative rate constants of Toc-3H homologues to α -Toc-3H in membranes ($\alpha/\beta/\gamma/\delta=100/83.7/63.2/20.2$) were not much different to those of TocH homologues to α -TocH ($\alpha/\beta/\gamma/\delta=100/88.4/83.8/17.3$). Each k_s value of Toc-3H homologues in membranes was 60–80% of that of the corresponding TocH homologues except for the almost identical k_s values of δ -homologues, but there was no difference in EtOH solution between each k_s value of the corresponding homologues of Toc-3H and TocH. These results indicate that the difference of the alkyl-side chain structure of vitamin E causes a change in the mobility of vitamin E molecules and/or the location of their antioxidant OH-groups in membranes, resulting in lowered radical-trapping rates of Toc-3Hs. By use of the ratio of the k_{inh} value of α -TocH with $\text{LOO}\cdot$ ($3.20 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$) to the k_s value of α -TocH with $\text{ArO}\cdot$ ($8.05 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$) in chlorobenzene (that is, 39.8), the k_{inh} value for the reaction of α -TocH with $\text{LOO}\cdot$ in membrane was estimated to be $1.03 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$.

Key Words vitamin E, tocopherol, tocotrienol, antioxidant, membrane

Vitamin E is a generic term for all tocopherol (TocH) and tocotrienol (Toc-3H) homologues. The antioxidant effect of vitamin E has been ascribed to the oxidation of its phenolic hydroxyl group by lipid peroxyl radicals ($\text{LOO}\cdot$), producing corresponding tocopheroxyl radicals (Toc $\cdot$) or tocotrienoxyl radicals (Toc-3 $\cdot$) (reaction 1). The mechanism involved has been studied extensively by several investigators (1–5).

It is well known that lipophilic vitamin E is localized in biomembranes and functions as an efficient inhibitor of lipid peroxidation in membranes (6–10). We recently reported the second-order rate constants (k_s) for the reactions of α -, β -, γ -, δ -TocH, and tocol with the 2,6-di-*t*-butyl-4-(4-methoxyphenyl)phenoxyl radical ($\text{ArO}\cdot$), which can be regarded as a model for lipid peroxyl radicals ($\text{LOO}\cdot$), in membranes (reaction 2) using stopped-flow spectrophotometry (10).

In this experiment, we used samples of egg yolk phosphatidylcholine (EYPC) vesicles as a membrane model in a 70% methanol solution with low turbidity, which is suitable for measuring by spectrophotometry. In the

present work, we measured k_s values in EYPC membranes for the reactions of α -, β -, γ -, and δ -Toc-3Hs with the $\text{ArO}\cdot$ radical.

Experimental Procedures

The α -, β -, γ -, δ -tocotrienols and α -tocopherols were kindly supplied by Eisai Food & Chemical Co., Ltd. (Tokyo, Japan). Egg yolk phosphatidylcholine (EYPC) was obtained from Nippon Oil and Fat Co. (Tokyo, Japan). The aroxyl radical ($\text{ArO}\cdot$) was synthesized as previously reported (11).

EYPC vesicles used as a membrane model were prepared in 70% MeOH (MeOH : H_2O = 7 : 3, vol/vol), because samples with low turbidity that maintain the membrane structure are suitable for the measurement of spectra (10). We prepared a 70% MeOH solution of $\text{ArO}\cdot$ just before measurement by stopped-flow spectrophotometry. The ratio of α -TocH to phospholipids in biological membranes is reported to be 1 : 1,000–2,000 (12). In the present work, measurements of the rate constants (k_s) were performed using membranes for which the ratios of Toc-3H to EYPC were 1 : 100–500, because the lower ratio of phospholipid to antioxidant produced a sample solution with the lower turbidity suitable for spectrophotometry.

The kinetic data were obtained with a Unisoku Model RS-450 stopped-flow spectrophotometer by mixing

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Fig. 1. (A) Changes in the electronic absorption spectra of $\text{ArO}^\bullet$ and $\alpha\text{-Toc-3}^\bullet$ during the reaction of $\text{ArO}^\bullet$ with $\alpha\text{-Toc-3H}$ in 10 mM EYPC vesicles in 70% MeOH solution at 25°C . The spectra were recorded at 8 ms intervals. The arrow indicates a decrease in the absorbance of $\text{ArO}^\bullet$ and an increase in the absorbance of $\alpha\text{-Toc-3}^\bullet$. $[\text{ArO}^\bullet]_{t=0} \sim 7.0 \times 10^{-5} \text{ M}$ and $[\alpha\text{-Toc-3H}]_{t=0} = 1.27 \times 10^{-4} \text{ M}$. (B) Time courses of the decrease of absorbance at 583 nm associated with the decay of $\text{ArO}^\bullet$ during the reaction of $\text{ArO}^\bullet$ with $\alpha\text{-Toc-3H}$ in 10 mM EYPC vesicles in 70% MeOH solution at 25°C . Concentrations of $\alpha\text{-Toc-3H}$ were (a) $1.27 \times 10^{-4} \text{ M}$, (b) $1.02 \times 10^{-4} \text{ M}$, (c) $7.63 \times 10^{-5} \text{ M}$, (d) $5.09 \times 10^{-5} \text{ M}$, (e) $2.54 \times 10^{-5} \text{ M}$. $[\text{ArO}^\bullet]_{t=0} \sim 7.0 \times 10^{-5} \text{ M}$.

equal volumes of 70% MeOH solutions of (A) 0.1 mM $\text{ArO}^\bullet$ and (B) 20 mM EYPC vesicles containing vitamin E under a nitrogen atmosphere.

The time between mixing the two solutions and recording the first data point (that is, dead time) was 10–20 ms. The reaction was monitored by either single wavelength detection or a photo-diode array detector attached to the stopped-flow spectrophotometer. All measurements were performed at $25.0 \pm 0.5^\circ\text{C}$. Experimental errors in the rate constants (k_s) are estimated to be about 5% and 8% in chlorobenzene and vesicle solutions, respectively.

Results and Discussion

The changes in the absorption spectrum measured during the reaction of $\alpha\text{-Toc-3H}$ with $\text{ArO}^\bullet$ (Eq. (3)) are shown in Fig. 1A. When a 70% MeOH solution of $\alpha\text{-Toc-3H}$ with EYPC vesicles was added to a 70% MeOH solution of $\text{ArO}^\bullet$, the $\text{ArO}^\bullet$ absorption peak disappeared immediately, the $\alpha\text{-Toc-3}^\bullet$ peak appeared, and the isosbestic points were observed. As shown in Fig. 1B, after about 5 ms lag time probably due to penetration of $\text{ArO}^\bullet$ into membranes of EYPC vesicles, the decay of absorbance of $\text{ArO}^\bullet$ (at 583 nm) was observed.

The pseudo-first-order rate constants (k_{obsd}) were determined by evaluating the decrease in the absorbance of the $\text{ArO}^\bullet$ by the reaction with various concentrations of $\alpha\text{-Toc-3H}$. As shown in Fig. 2, the k_{obsd} of $\text{ArO}^\bullet$ at 583 nm plotted against $\alpha\text{-Toc-3H}$ concentrations was linearly dependent on the $\alpha\text{-Toc-3H}$ concentration, and thus the rate equation was expressed as

$$-\frac{d[\text{ArO}^\bullet]}{dt} = k_{\text{obsd}} [\text{ArO}^\bullet] = k_s [\alpha\text{-Toc-3H}] [\text{ArO}^\bullet] \quad (3)$$

where k_s is the second-order rate constant for oxidation of $\alpha\text{-Toc-3H}$ by $\text{ArO}^\bullet$. Similar measurements were performed for β -, γ -, and δ -Toc-3Hs (Fig. 2).

We calculated their membranous second-order rate

Fig. 2. Dependence of pseudo-first-order rate constant (k_{obsd}) values on concentration of Toc-3H homologues in 10 mM EYPC vesicles in 70% MeOH solution at 25°C . The k_{obsd} values of $\alpha\text{-Toc-3H}$ were obtained by analyzing the decrease in the absorbance of $\text{ArO}^\bullet$ at 583 nm, as shown in Fig. 1B. The k_{obsd} values of the other Toc-3H homologues were obtained from the time courses of the decrease of absorbance at 583 nm associated with the decay of $\text{ArO}^\bullet$ during the reaction with Toc-3H homologues (data not shown). ●, $\alpha\text{-Toc-3H}$; ○, $\beta\text{-Toc-3H}$; ▲, $\gamma\text{-Toc-3H}$; △, $\delta\text{-Toc-3H}$.

constant (k_s) assuming that all antioxidant molecules were located in EYPC membranes and using the estimation that their concentration in EYPC membranes (mol of Toc-3H or TocH/L of EYPC) was 133 times higher in the bulk MeOH/H₂O phase (13). The k_s values of Toc-3H and TocH homologues obtained in EYPC membrane and in EtOH solution are summarized in Table 1. The k_s values of Toc-3H and TocH homologues

Table 1. Second-order rate constants (k_s) for reaction of $\text{ArO}\cdot$ with Toc-3Hs and TocHs in EYPC vesicles and EtOH.

	EYPC vesicle (observed) $k_s (\text{M}^{-1}\text{s}^{-1})$		EYPC vesicle (corrected) ¹ $k_s (\text{M}^{-1}\text{s}^{-1})$		EtOH $k_s (\text{M}^{-1}\text{s}^{-1})$	
	Toc-3H	TocH ²	Toc-3H	TocH ²	Toc-3H ³	TocH ^{2,3}
α -Form	2.78×10^5	3.45×10^5	2.09×10^3	2.59×10^3	4.97×10^3	5.12×10^3
β -Form	2.33×10^5	3.04×10^5	1.75×10^3	2.29×10^3	3.04×10^3	2.77×10^3
γ -Form	1.75×10^5	2.88×10^5	1.32×10^3	2.17×10^3	2.76×10^3	2.82×10^3
δ -Form	5.63×10^4	5.95×10^4	4.23×10^2	4.48×10^2	1.09×10^3	1.02×10^3

¹ Corrected k_s values were calculated by adjusted concentrations of Toc-3H and TocH in membranes (assumed to be 133 times concentrated) (13).

² k_s values reported in Ref. (10).

³ k_s values reported in Ref. (16).

Table 2. Relative rate constants (k_s) for reaction of $\text{ArO}\cdot$ with Toc-3Hs and TocHs ($100k_s(\text{Toc-3H})/k_s(\alpha\text{-Toc-3H})$ and $100k_s(\text{TocH})/k_s(\alpha\text{-TocH})$), and comparison of rate constants between each analogue of Toc-3H and TocH ($100k_s(\text{Toc-3H})/k_s(\text{TocH})$) in EYPC vesicles and EtOH.

	EYPC vesicle		EtOH		$100k_s(\text{Toc-3H})/k_s(\text{TocH})$	
	$100k_s(\text{Toc-3H})/k_s(\alpha\text{-Toc-3H})$	$100k_s(\text{TocH})/k_s(\alpha\text{-TocH})$	$100k_s(\text{Toc-3H})/k_s(\alpha\text{-Toc-3H})$	$100k_s(\text{TocH})/k_s(\alpha\text{-TocH})$	EYPC vesicle	EtOH
α -Form	100	100	100	100	80.6	97.1
β -Form	83.7	88.4	61.2	54.1	76.6	109.7
γ -Form	63.2	83.8	55.5	55.1	60.8	97.9
δ -Form	20.2	17.3	21.9	19.9	94.6	106.9

Table 3. Second-order rate constants (k_s and k_{inh}) for reaction of $\text{ArO}\cdot$ and $\text{LOO}\cdot$ with $\alpha\text{-TocH}$ (and $\alpha\text{-Toc-3H}$) in chlorobenzene (and EYPC vesicles), respectively.

	Chlorobenzene		$k_{\text{inh}}(\text{LOO}\cdot)/k_s(\text{ArO}\cdot)$	EYPC vesicle	
	$k_s(\text{ArO}\cdot)$ ($\text{M}^{-1}\text{s}^{-1}$)	$k_{\text{inh}}(\text{LOO}\cdot)$ ($\text{M}^{-1}\text{s}^{-1}$)		$k_s(\text{ArO}\cdot)$ ($\text{M}^{-1}\text{s}^{-1}$)	$k_{\text{inh}}(\text{LOO}\cdot)$ ($\text{M}^{-1}\text{s}^{-1}$)
$\alpha\text{-TocH}$	8.05×10^4 (observed) ¹	3.20×10^6 (reported) ²	39.8	2.59×10^3 (observed) ¹	1.03×10^5 (estimated) ³
$\alpha\text{-Toc-3H}$	8.09×10^4 (observed) ¹	3.22×10^6 (estimated) ³	39.8	2.09×10^3 (observed) ¹	8.31×10^4 (estimated) ³

¹ k_s values measured by this experiment.

² k_s values reported in Ref. (1).

³ k_s values estimated by using the value of the ratio ($k_{\text{inh}}(\text{LOO}\cdot)/k_s(\text{ArO}\cdot)=39.8$) (see text).

in membrane were about half of those in EtOH solution. The k_s values of Toc-3H homologues in membrane increased in the order of $\delta\text{-Toc-3H} < \gamma\text{-Toc-3H} < \beta\text{-Toc-3H} < \alpha\text{-Toc-3H}$. This order was same as that of the k_s values of TocH homologues in membrane reported recently by us (10). Furthermore, the relative reaction rate constants of Toc-3H analogues to $\alpha\text{-Toc-3H}$ in membrane ($\alpha/\beta/\gamma/\delta=100/83.7/63.2/20.2$) were not much different to those of the reaction rate constants of TocH homologues to $\alpha\text{-TocH}$ in membrane ($\alpha/\beta/\gamma/\delta=100/88.4/83.8/17.3$) (Table 2), indicating that the relative radical trapping activities of Toc-3H homologues in membrane are almost the same as those of

TocH homologues.

Yoshida et al. (14) reported that the reactivities of α -, β -, γ -, and δ -Toc-3Hs toward peroxy radicals are the same as those of the corresponding α -, β -, γ -, and δ -Toc-3H homologues in acetonitrile solution. On the other hand, Servinova et al. (15) reported that $\alpha\text{-Toc-3H}$ has more potent antioxidant activity than $\alpha\text{-TocH}$ for lipid peroxidation in rat liver microsomal membranes. In the present work, each k_s value of Toc-3H homologues in membranes was 60–80% of that of the corresponding TocH homologue except for the nearly identical k_s values of δ -homologues, although there was no difference in EtOH solution between each k_s value of corresponding

homologues of Toc-3H and TocH (16) (Table 2). These results indicate that the difference in the alkyl side chain structure of vitamin E causes the change in the mobility of vitamin E molecules and/or the location of their antioxidant OH-groups in membranes, resulting in the lowered radical-trapping rate of Toc-3H.

Next, we tried to evaluate the k_{inh} value for the reaction of α -TocH with $LOO\cdot$ in membrane, by assuming that the ratio of the k_{inh} value of α -TocH with $LOO\cdot$ to the k_s value with $ArO\cdot$ in solvent is the same as their ratio (k_{inh}/k_s) in membranes. We previously reported that the k_s value of α -TocH was dependent on the polarity of the solvents and increased with a decrease in the polarity of solvent (4).

In fact, the k_s values (8.09×10^4 and $8.05 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$) obtained for α -Toc-3H and α -TocH in chlorobenzene were larger than the corresponding ones (4.97×10^3 and $5.12 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$) in EtOH with higher polarity (see Table 3). Since the k_{inh} value of α -TocH in chlorobenzene was reported to be $3.20 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$ (1), we compared this k_{inh} value to the k_s value ($8.05 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$) of α -TocH in chlorobenzene.

By use of the value of the ratio ($k_{inh} (LOO\cdot)/k_s (ArO\cdot) = 39.8$) in chlorobenzene, the k_{inh} value of α -TocH in membrane was tentatively estimated to be $1.03 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ ($= 39.8 \times 2.59 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$), where $2.59 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$ is the k_s value for reaction of α -TocH with $ArO\cdot$ in membranes (see Table 3). The k_{inh} value ($1.03 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$) estimated was about twice the k_{inh} value in 1-palmitoyl-2-linoleoylphosphatidylcholine membranes ($4.7 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$) reported by Barclay et al. (7), suggesting higher mobility of EYPC molecules in membranes prepared in 70% MeOH solution.

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