Rate Constants for Hydrogen Atom Abstraction by α-Tocopheroxyl Radical from Lipid, Hydroperoxide and Ascorbic Acid

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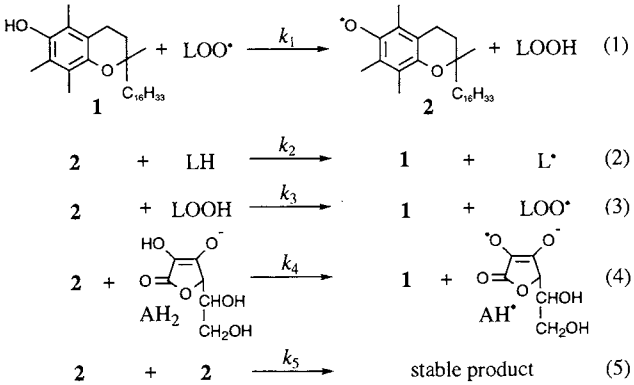
The rate constants for each reaction of α-tocopheroxyl radical with methyl linoleate, *t*-butyl hydroperoxide and ascorbic acid, and also the bimolecular reaction of α-tocopheroxyl radicals were measured by a stopped-flow ESR technique in order to understand the antioxidant action of α-tocopherol. It was suggested that α-tocopheroxyl radical is primarily reduced by ascorbic acid *in vivo*.

There is now an increasing experimental and clinical evidence which suggests the involvement of active oxygen species and free radicals in the pathogenesis of various diseases, cancer and aging. Consequently, the role of antioxidants has received much attention. α-Tocopherol, the most active form of vitamin E, acts as a lipophilic chain-breaking antioxidant *in vivo*.[1] It scavenges active radicals such as peroxyl radicals rapidly to inhibit lipid peroxidation. The potency of an antioxidant is determined not only by its reactivity toward radicals but also by the fate of the antioxidant-derived radical. When α-tocopherol **1** scavenges peroxyl radical, it gives α-tocopheroxyl radical **2** (reaction 1). α-Tocopheroxyl radical **2** may undergo several reactions. It may scavenge another peroxyl radical LOO• to give an adduct,[2,3] or attack lipids LH (reaction 2) and hydroperoxides LOOH (reaction 3) to give lipid and lipid peroxyl radicals respectively, which are capable of inducing lipid peroxidation. Under these circumstances, the antioxidant effect of α-tocopherol is diminished. On the other hand, **2** may be reduced by ascorbate to regenerate α-tocopherol **1** (reaction 4). It may also undergo bimolecular interaction with another **2** to give a stable product (reaction 5). The relative importance of these competing



reactions depends on the circumstances and determines the activity of α-tocopherol as an antioxidant.[4] Therefore, to know the rate constants for such reactions is of prime importance for understanding the role of vitamin E. Since α-tocopheroxyl radical **2** decays rapidly, the rate constants for the reaction 2 - 4 have been difficult to be measured, although those for more stable, relevant chromanoxyl radicals have been measured.[5] The objective of the present study was to measure the rate constants for the reactions 2, 3, 4 and 5 by a stopped-flow ESR technique.

Natural 2R, 4'R, 8'R-α-tocopherol was kindly supplied by Eisai Co., Ltd. (Tokyo). Commercial methyl linoleate was purified before use as reported previously.[6] Ascorbic acid and *t*-butyl hydroperoxide were obtained from Sigma Chemical Co. (St. Louis, Mo). Other chemicals were those of the highest grade available commercially. ESR measurements were performed at 37 °C in ethanol unless otherwise stated with an X-band ESR spectrometer, JES-TE100, equipped with a rapid-mixer, ES-SM2 (JEOL, Tokyo). Nitrogen gas was bubbled through the ethanol solution to remove air before ESR recording.

When excess α-tocopherol was reacted with galvinoxyl, the ESR spectrum of galvinoxyl disappeared instantaneously and a new ESR spectrum characteristic of α-tocopheroxyl radical appeared. The time which elapsed between the mixing of reactants and the beginning of recording operations (~ 1 s) corresponded to full consumption of galvinoxyl. It was confirmed that the relative ESR signal intensity was proportional to the galvinoxyl concentration. The α-tocopheroxyl radical decayed rapidly, the rate being dependent on its initial concentration. For example, when 25 μM galvinoxyl and 500 μM α-tocopherol were mixed, the half-life of α-tocopheroxyl radical was about 25 s. The magnetic field was set constant at 334.540 mT and its decay with time was followed continuously. Under these conditions, the decay of α-tocopheroxyl radical is given by Eq. (6).

$$-d[\mathbf{2}] / dt = k_5[\mathbf{2}]^2 \quad (6)$$

Hence,

$$1 / [\mathbf{2}] = k_5 t + [\mathbf{2}]_0^2 \quad (7)$$

where k_5, t and $[\mathbf{2}]_0$ are the rate constant for bimolecular interaction of **2**, time and the initial concentration of **2** respectively. The plot of $1/[\mathbf{2}]$ as a function of time gave a straight line and the rate constant k_5 was obtained from the slope as 1.0×10^3 $M^{-1} s^{-1}$, which is in good agreement with the literature values.[7] In the presence of substrate **S**, α-tocopheroxyl radical decays with the rate law:

$$-d[\mathbf{2}] / dt = k_5[\mathbf{2}]^2 + k_a[\mathbf{2}][\mathbf{S}] \quad (8)$$

where k_a is the rate constant for the reaction between **2** and **S**. Equation 9 is obtained by an integration of Eq. 8, where $[\alpha\text{-TO}^\bullet]$ and $[\alpha\text{-TO}^\bullet]_0$ denote the concentrations of **2** at time t and 0 respectivety. The experimental decay curve for α-tocopheroxyl radical in the presence of methyl linoleate is shown as an example in Figure 1. Galvinoxyl was depleted by the reaction with excess α-tocopherol before introducing a substrate. The formation of α-tocopherol from α-tocopheroxyl

Table 1. Rate of reactions of α-tocopheroxyl radical **2**

Reaction	Rate constant[a] $M^{-1}s^{-1}$	Concentration M	Rate M/s
2 + LH → 1 + L•	3×10^{-2}	[LH] = 1	3×10^{-9}
2 + LOOH → 1 + $LO_2^\bullet$	4×10^{-1}	[LOOH] = 10^{-8}	4×10^{-16}
2 + AH_2 → 1 + AH•	1×10^{5}	[AH_2] = 10^{-4}	1×10^{-6}
2 + LOO• → stable product	10^{8}	[LOO•] = 10^{-10}	10^{-9}
2 + 2 → stable product	1×10^{3}	[2] = 10^{-7}	1×10^{-11}

[a] Obtained in this study except for the reaction with peroxyl radical which was assumed to be 10^8 $M^{-1}s^{-1}$.[8]

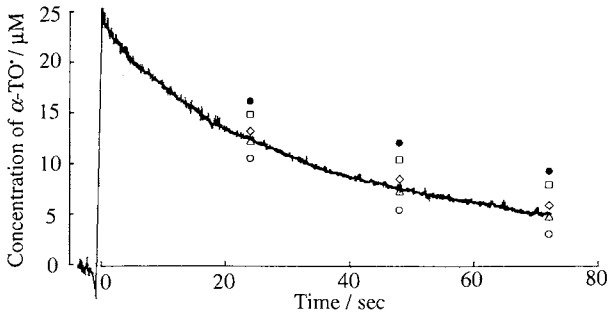


Figure 1. An experimental decay curve of α-tocopheroxyl radical in the presence of methyl linoleate (376 mM) in ethanol at 37°C under nitrogen, [α-tocopherol] = 500 μM, [galvinoxyl] = 25 μM. The points □, ◇, △ and ○ show the concentrations calculated by assuming the value of k_2 as 0.005, 0.02, 0.03 and 0.05 $M^{-1}s^{-1}$ respectively. The points ● show experimental data in the absence of methyl linoleate.

$$[\alpha\text{-TO}^{\cdot}] = \frac{\dfrac{k_a[\alpha\text{-TO}^{\cdot}]_0}{k_5[\alpha\text{-TO}^{\cdot}]_0 + k_a} e^{-k_a t}}{1 - \dfrac{k_5[\alpha\text{-TO}^{\cdot}]_0}{k_5[\alpha\text{-TO}^{\cdot}]_0 + k_a} e^{-k_a t}} \quad (9)$$

radical by the reaction with **S** was confirmed by HPLC. The calculated curves obtained by assuming various values for the rate constant k_2 are also included in Figure1. Thus, the rate constants for the reactions of α-tocopheroxyl radical with methyl linoleate, *t*-butyl hydroperoxide and ascorbic acid were obtained as summarized in Table 1.

The relevant rate constants have been reported by several groups. Remorova and Roginskii[8] measured the rate constant for hydrogen atom abstraction from methyl linoleate by α-tocopheroxyl radical under steady state condition with ESR and obtained a value 0.075 $M^{-1}s^{-1}$ at 50 °C in benzene. Mukai *et al.* have measured numerous rate constants for more stable tocopheroxyl radical, for example, they obtained values of 0.018, 0.365 and 0.133 $M^{-1}s^{-1}$ for hydrogen atom abstraction from ethyl linoleate[9], *t*-butyl hydroperoxide[10] and methyl linoleate hydroperoxide[11] by 5,7-diisopropyl tocopheroxyl radical at 25 °C in benzene respectively. The rate constants for α-tocopheroxyl radical could not be measured accurately due to its rapid decay. As for the reaction of α-tocopheroxyl radical with ascorbate, Packer *et al.*[12] and Scarpa *et al.*[13] determined the rate constant as 1.55×10^6 $M^{-1}s^{-1}$ at 25 °C in isopropyl alcohol/acetone/water and 2×10^5 $M^{-1}s^{-1}$ at 22 °C in phosphatidylcholine liposomal membranes respectively, but the decay by bimolecular interaction was not considered in either of the studies.

The rate constants obtained in this study enable us to estimate the relative importance of the competing reactions for α-tocopheroxyl radical. The physiological concentrations of polyunsaturated lipid, lipid hydroperoxide, α-tocopherol and ascorbic acid were assumed to be 1, 10^{-8}, 10^{-5} and 10^{-4} M.[14] The concentrations of peroxyl radical and α-tocopheroxyl radical were calculated from the following equations,

$$[LO_2^{\cdot}] = R_i / 2\,k_{inh}[\mathbf{1}] \quad (10)$$

$$[\mathbf{2}] = k_{inh}[\mathbf{1}] / k_6 \quad (11)$$

where R_i, k_{inh} and k_6 are the rate of chain initiation and the rate constants for the reactions between **1** and peroxyl radical, and between **2** and peroxyl radical respectively.[15,16] The rate constants k_{inh} and k_6 were assumed to be 10^6 and 10^8 $M^{-1}s^{-1}$. The rate of chain initiation was assumed to be 2×10^{-9} M/s. The concentration of **2** is calculated from Eq. 11 as 10^{-7} M by assuming [**1**] as 10^{-5} M. The results shown in Table 1 suggest that α-tocopheroxyl radical is primarily reduced by ascorbic acid under physiological conditions. The reaction of **2** with lipid hydroperoxide or the bimolecular reactions may not be important. The concentration of peroxyl radical is proportional to the rate of chain initiation. The rate of free radical flux *in vivo* is not known, but the reaction between **2** and peroxyl radical may become important only at high rate of radical flux. In conclusion, it may be said that α-tocopheroxyl radical **2** is primarily reduced by ascorbic acid under physiological conditions[17] and that the attack of **2** upon polyunsaturated lipids may become important in the absence of ascorbic acid.

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17 Ubiquinol may also contribute to the reduction of α-tocopheroxyl radical where its concentration is high such as in mitochondria.