Tunneling Effect in Regeneration Reaction of Vitamin E by Ubiquinol

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A kinetic study of the regeneration reaction of vitamin E by ubiquinol was carried out by means of double-mixing stopped-flow spectroscopy. A substantial deuterium kinetic-isotope effect was observed on the second-order rate constant and the activation energy. In the regeneration reaction of α-tocopherol, deuteration of ubiquinol increased and decreased the activation energy and the second-order rate constant by 6.1 kJ/mol and a factor of 18.3, respectively. From this result, it is considered that proton tunneling plays an important role in the regeneration reaction of vitamin E by ubiquinol. The conditions under which the tunneling effect becomes an important factor were discussed in conjunction of our experimental results.

1. Introduction

It is well-known that ubiquinone has an important function as an electron carrier in the mitochondrial respiratory chain.[1−3] Mitochondria are rich in unsaturated lipid[4] and must be protected against peroxidation. In spite of recent criticism,[5] the traditional and most widely accepted view[6] is that vitamin E (TocH) prevents peroxidation in the biomembrane (reaction 1 and Figure 1) by scavenging the lipid peroxyl radical (LOO•).[7−10]

$$\mathrm{TocH} + \mathrm{LOO\bullet} \rightarrow \mathrm{Toc\bullet} + \mathrm{LOOH} \qquad (1)$$

where Toc• and LOOH denote a tocopheroxyl radical and a lipid hydroperoxide, respectively.

Several investigators found that the reduced form of ubiquinone (ubiquinol, UQH_2) also has strong activity in inhibiting the lipid peroxidation in various mammalian organisms such as liver mitochondria, bovine heart submitochondria, rat liver, and human low-density lipoprotein.[3,11−14] It is considered that UQH_2 prevents the lipid peroxidation in biomembrane (Figure 1)[2,3,15−22] by scavenging LOO• (reaction 2) and by regenerating TocH (reaction 3) from Toc•, which has been produced through reaction 1.

$$\mathrm{UQH_2} + \mathrm{LOO\bullet} \rightarrow \mathrm{UQH\bullet} + \mathrm{LOOH} \qquad (2)$$

$$\mathrm{UQH_2} + \mathrm{Toc\bullet} \rightarrow \mathrm{UQH\bullet} + \mathrm{TocH} \qquad (3)$$

where UQH• refers to a dehydroubiquinol radical. The TocH regenerated through reaction 3 can contribute again to reaction 1 and prevents the peroxidation in the biomembrane by scavenging LOO• (Figure 1). In fact, TocH and UQH_2 coexist in relatively high concentrations in plasma, various tissues, and skin.[23−26] Toc•'s other than those transformed into TocH's through some regeneration reactions such as reaction 3 may react with lipid (LH) to produce lipid radical (L•) leading to LOO• (prooxidant reaction, Figure 1), which is dangerous to the biomembrane.[27]

As mentioned above, UQH_2 is the reduced form of ubiquinone. Ubiquinone predominantly found in human cells is ubiquinone-10 (coenzyme Q10),[28] although ubiquinone homologues containing various isoprene units occur in nature, and some ubiquinones having short isoprenoid-tail lengths are found in microorganisms.[29] In human plasma and various tissues except brain and lung,[2,3,30−33] ubiquinol-10 (reduced form, $UQ_{10}H_2$, Figure 2) is superior to ubiquinone-10 (oxidized form) in concentration, and so $UQ_{10}H_2$ is considered to prevent the lipid peroxidation. In this paper, we focus our attention on reaction 3 for $UQ_{10}H_2$ (reaction 4).

$$\mathrm{UQ_{10}H_2} + \mathrm{Toc\bullet} \xrightarrow{k_{UQ}} \mathrm{UQ_{10}H\bullet} + \mathrm{TocH} \qquad (4)$$

It is well-known that hydrophilic vitamin C (ascorbate monoanion, AsH^-) also regenerates TocH from Toc• at the interface between biomembrane and water phase (reaction 5, Figure 1).[34−37]

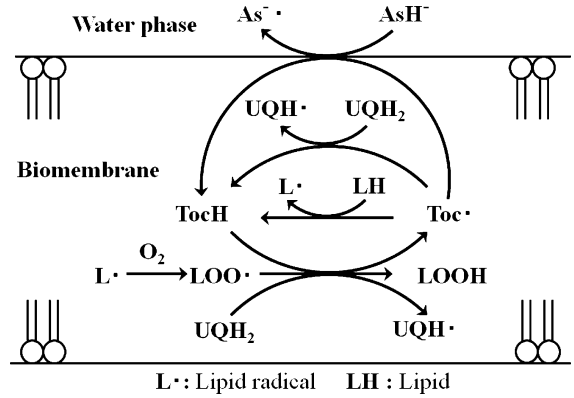

Figure 1. Partial scheme of LOO• production and of the antioxidant, prooxidant, and regeneration reactions of TocH in a biomembrane.

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$$AsH^- + Toc\bullet \xrightarrow{k_{VC}} As^-\bullet + TocH \qquad (5)$$

where $As^-\bullet$ denotes an ascorbate monoanion radical. However, reaction 4 has a rate constant comparable, within about 1 order of magnitude, to that of reaction 5.[17,19] Furthermore, $UQ_{10}H_2$ is found in relatively high concentration in human plasma and various tissues,[3,23,25,30−33] and lipophilic $UQ_{10}H_2$ can react with Toc• inside the biomembrane, in contrast to hydrophilic AsH^- existing in the water phase (Figure 1). Like this, reaction 4 has some advantages to the regeneration of TocH in vivo.

In previous studies,[38,39] by using stable 5,7-diisopropyl-Toc• (Figure 2), we carried out kinetic studies of reaction 4, and by substituting deuterons for the hydroxy protons in $UQ_{10}H_2$ ($UQ_{10}D_2$, Figure 2) we observed a substantial deuterium kinetic-isotope effect on the second-order rate constant and the activation energy. This result suggested that the tunneling effect plays an important role in reaction 4, which is a proton (or hydrogen) transfer reaction from $UQ_{10}H_2$ to Toc•. In a recent paper, Kikuta et al[40] suggested that what we refer to as the "tunneling effect" is more accurately described as a "quantum effect." For consistency with previous usage, however, in this paper we use the designation "proton tunneling effect."

Similarly to the case of reaction 4 for 5,7-diisopropyl-Toc•, the proton tunneling effect plays an important role in reaction 5 (TocH-regeneration by AsH^-) for 5,7-diisopropyl-Toc•.[39] However, the tunneling does not contribute to reaction 5 for α- and β-Toc•'s originating from natural α- and β-TocH's (Figure 2).[37] Accordingly, it is essential to study the kinetics of reaction 4 for natural Toc• and to examine whether the tunneling effect really contributes to reaction 4 in vivo. Furthermore, it is very important to clarify the conditions under which the tunneling effect becomes an important factor, and it is also necessary to elucidate the reason why the proton tunneling occurs under those conditions. For example, why does the structure of Toc• influence the presence (or absence) of the tunneling effect in reaction 5 as noted above?

In the work presented here we have studied the regeneration reaction of natural TocH by $UQ_{10}H_2$ (reaction 4) with a double-mixing stopped-flow spectrophotometer, which allows us to follow kinetics of reactions of short-lived radicals such as α- and β-Toc•'s. We have determined the second-order rate constants and the activation energies of reaction 4 and examined the deuterium kinetic-isotope effects. From these results, we have clarified the conditions under which the tunneling effect plays an important role in the regeneration reaction of TocH.

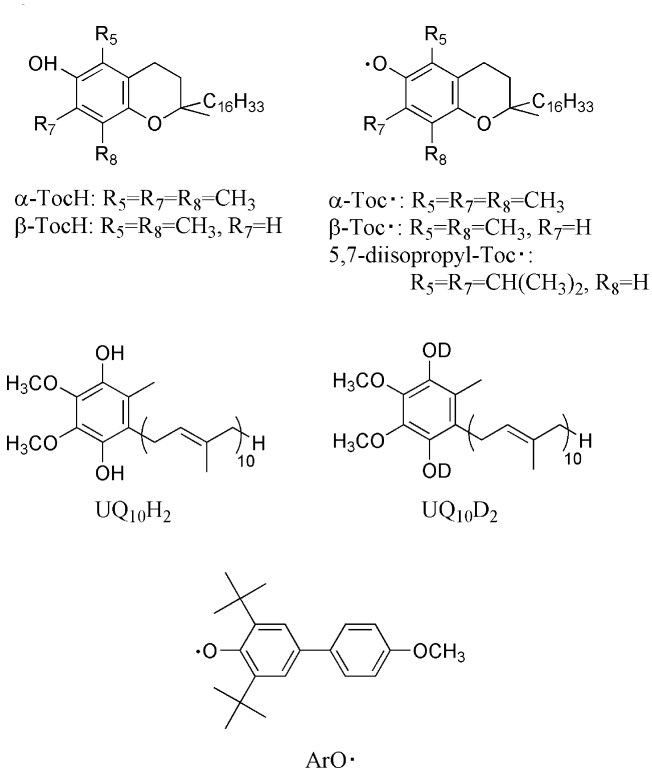

Figure 2. Molecular structures of TocH's, Toc•'s, $UQ_{10}H_2$, $UQ_{10}D_2$, and ArO•.

2. Experimental Methods

The structures of the molecules used in this work are shown in Figure 2. α- and β-TocH's were kindly supplied by Eisai Co., Ltd. and used without further purification. $UQ_{10}H_2$ was prepared by the reduction of ubiquinone-10 with sodium hydrosulfite under a nitrogen atmosphere.[22] Ubiquinone-10 was kindly supplied by Kaneka Corporation. We prepared 2,6-di-*t*-butyl-4-(4′-methoxyphenyl)phenoxyl radical (ArO•) as reported in a previous paper.[41] Ethanol (C_2H_5OH, EtOH) was obtained from Wako, dried over magnesium ribbon, and purified by distillation. Ethanol-d_1 (C_2H_5OD, EtOD) of 99.5% purity was purchased from Aldrich and used without purification. When $UQ_{10}H_2$ or TocH was dissolved in EtOD, replacement of the hydrogen atom(s) of the OH group(s) by (a) deuteron(s) was easily accomplished, and the deuterated molecule ($UQ_{10}D_2$ or TocD) was obtained. The deuteration was verified by proton NMR.

The kinetic data were obtained by using a Unisoku RSP-1000-03F double-mixing stopped-flow spectrophotometer under a nitrogen atmosphere at 15−40 °C. The error in the temperature reading was less than 0.5 °C. First Toc• radical (α- or β-Toc•, Figure 2) was prepared by mixing equal volumes of EtOH (EtOD) solutions of ArO• and the TocH (TocD) (reaction 6) in the first-mixing unit of the stopped-flow spectrophotometer.

$$ArO\bullet + TocH(TocD) \rightarrow ArOH(ArOD) + Toc\bullet \qquad (6)$$

The concentrations of α-TocH, α-TocD, β-TocH, and β-TocD were 7.38×10^{-4}, 6.76×10^{-4}, 2.54×10^{-3}, and 1.25×10^{-2} M, respectively. After 2−30 s (5 s in α-TocH, 30 s in α-TocD, and 2 s in β-TocH and β-TocD), equal volumes of the resultant mixture and an EtOH (EtOD) solution of $UQ_{10}H_2$ ($UQ_{10}D_2$) were mixed [reaction 4-H (4-D)] in the second mixing unit.[37]

$$UQ_{10}H_2 + Toc\bullet \xrightarrow{k_{UQ}^{H}} UQ_{10}H\bullet + TocH \qquad (4\text{-H})$$

$$UQ_{10}D_2 + Toc\bullet \xrightarrow{k_{UQ}^{D}} UQ_{10}D\bullet + TocD \qquad (4\text{-D})$$

Reaction 4-H (4-D) was studied under pseudo-first-order conditions ($[UQ_{10}H_2(UQ_{10}D_2)] \gg [Toc\bullet]$, where the brackets [ ] indicate molar concentration). The prepared $[UQ_{10}H_2(UQ_{10}D_2)]$ was chosen so that the [Toc•] would not largely decrease from the initial concentration within the time required to completely mix $UQ_{10}H_2$ ($UQ_{10}D_2$) with Toc• and to make the solution homogeneous.

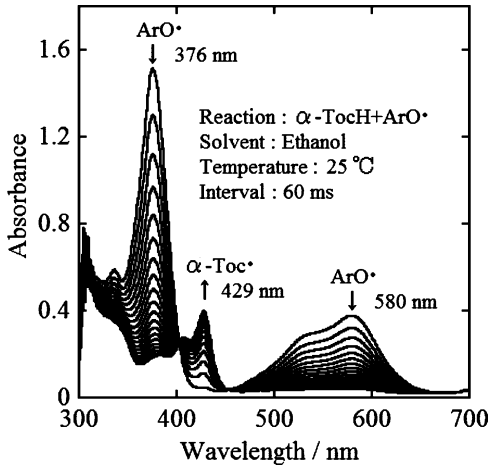

Figure 3. Change in absorption spectrum (at 60 ms intervals) during reaction of α-TocH and ArO• in EtOH at 25 °C (reaction 6). The prepared [α-TocH] was 0.738 mM. The arrows indicate a decrease and an increase in absorbance of ArO• and α-Toc•, respectively.

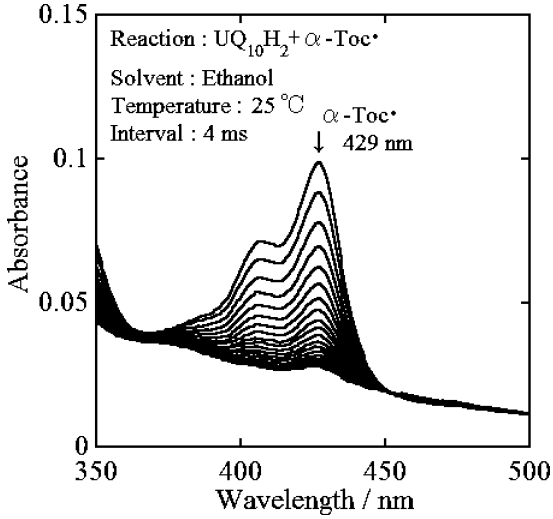

Figure 4. Change in absorption spectrum (at 4 ms intervals) during reaction of $UQ_{10}H_2$ and α-Toc• in EtOH at 25 °C (reaction 4-H). The prepared [$UQ_{10}H_2$] was 0.237 mM. The arrow indicates a decrease in absorbance of α-Toc•.

3. Results and Discussion

The change in absorption spectrum measured during reaction 6 for α-TocH is shown in Figure 3. Although ArO• was stable in the absence of α-TocH, when an EtOH solution with excess α-TocH was added to the ArO• solution, the ArO• absorption peak disappeared immediately, an α-Toc• peak appeared, and the isosbestic points were observed. The change in absorption spectrum measured during reaction 4-H for α-Toc• is shown in Figure 4, where the α-Toc• absorption peak disappears over time. $UQ_{10}H$• is so short-lived that the peak is not seen in the spectrum. Figures 5a and 5b show the absorbance decay of α-Toc• during reactions 4-H and 4-D in EtOH and EtOD, respectively. The absorbance decay of α-Toc• was well-characterized as a single-exponential decay, indicating that the bimolecular reaction of α-Toc•[42] is negligibly slower than reactions 4-H and 4-D. The absorbance decay was accelerated as the prepared [$UQ_{10}H_2(UQ_{10}D_2)$] increased, and the decay of reaction 4-H (Figure 5a) was much faster than that of reaction 4-D (Figure 5b) when [$UQ_{10}H_2$] ≅ [$UQ_{10}D_2$]. Similar results were also obtained for β-Toc•. γ- and δ-Toc•'s were so short-lived that their absorbance decays were not observed under our experimental conditions.

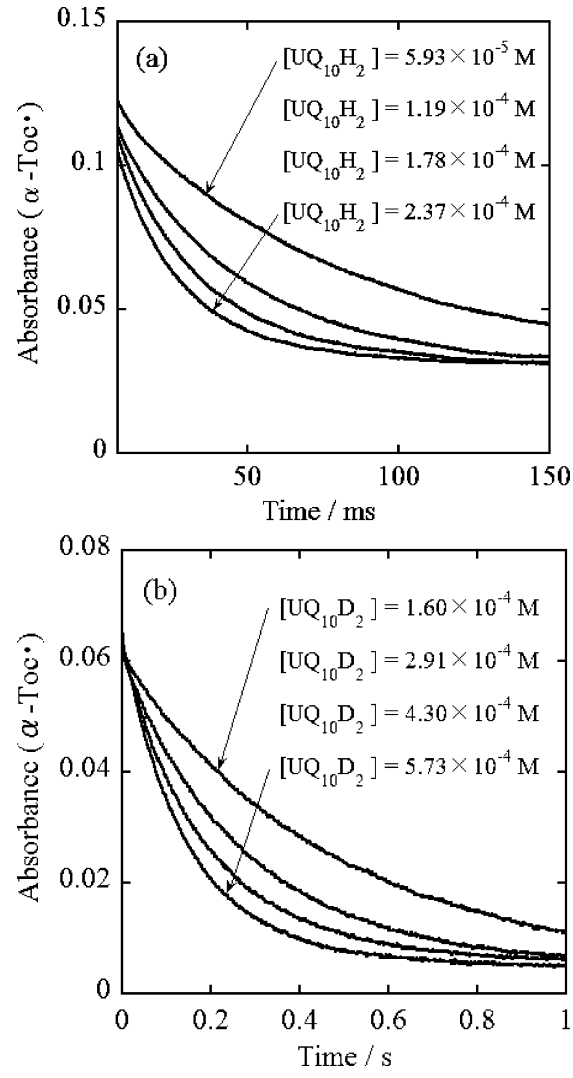

Figure 5. (a) Absorbance decay of α-Toc• at 429 nm at 25 °C during reaction 4-H in EtOH. (b) The absorbance decay during reaction 4-D in EtOD.

The pseudo-first-order rate constant (k_{obsd}) of reaction 4 was evaluated by using a standard linear least-squares analysis for each of the single-exponential absorbance decay-curves of α- and β-Toc•'s. The experimental equation is given as

$$-\mathrm{d[Toc\bullet]}/\mathrm{d}t = k_{obsd}[\mathrm{Toc\bullet}] \quad (7)$$

The rate equation of reaction 4 is expressed as

$$-\mathrm{d[Toc\bullet]}/\mathrm{d}t = (k_O + k_{UQ})[UQ_{10}H_2(UQ_{10}D_2)][\mathrm{Toc\bullet}] \quad (8)$$

where k_0 stands for the first-order rate constant for the natural decay of Toc• in EtOH (EtOD), and k_{UQ} is the second-order rate constant of reaction 4. k_{obsd} in eq 7 is given by

$$k_{obsd} = k_0 + k_{UQ}[UQ_{10}H_2(UQ_{10}D_2)] \quad (9)$$

where k_0 was much less than k_{UQ} [$UQ_{10}H_2(UQ_{10}D_2)$] under our experimental conditions. Since [$UQ_{10}H_2(UQ_{10}D_2)$] was nearly constant during reaction 4 under pseudo-first-order conditions ([$UQ_{10}H_2(UQ_{10}D_2)$] ≫ [Toc•]), k_{UQ} was evaluated from the slope

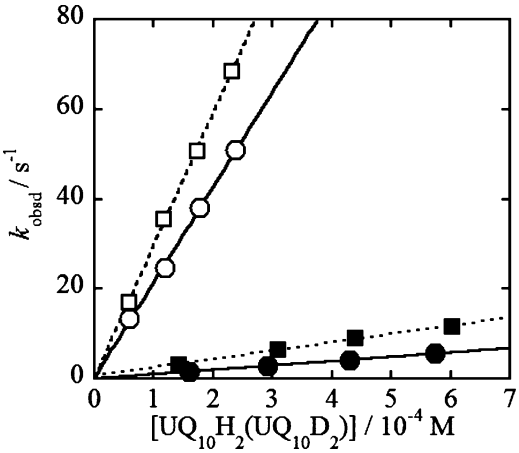

Figure 6. Dependence of k_{obsd} on prepared [$UQ_{10}H_2$($UQ_{10}D_2$)] at 25 °C in reactions of $UQ_{10}H_2$ and α-Toc• in EtOH (open circles), of $UQ_{10}D_2$ and α-Toc• in EtOD (filled circles), of $UQ_{10}H_2$ and β-Toc• in EtOH (open squares), and of $UQ_{10}D_2$ and β-Toc• in EtOD (filled squares) (reaction 4).

TABLE 1: k_{UQ} Values of Reaction 4 at 15, 20, 25, 30, 35, and 40 °C

	$10^{-4}k_{UQ}$ (M^{-1} s^{-1})					
reaction 4	15 °C	20 °C	25 °C	30 °C	35 °C	40 °C
$UH_{10}H_2$ + α-Toc•	19.9	20.6	21.2	21.7	22.5	23.4
$UH_{10}D_2$ + α-Toc•	0.93	1.09	1.16	1.25	1.30	1.41
$UH_{10}H_2$ + β-Toc•	20.6	24.0	29.5	36.0	42.5	52.4
$UH_{10}D_2$ + β-Toc•	1.03	1.37	1.85	2.75	3.70	4.73

of the plot of k_{obsd} versus the prepared [$UQ_{10}H_2$($UQ_{10}D_2$)] (Figure 6). The k_{UQ} values of reaction 4 for α- and β-Toc•'s are listed in Table 1.

It should be noted that the [$UQ_{10}H_2$] range employed in the evaluation of k_{UQ} was lower than the [$UQ_{10}D_2$] range (Figure 6). The k_{UQ} value for $UQ_{10}H_2$ (k_{UQ}^H) is much larger than that for $UQ_{10}D_2$ (k_{UQ}^D) (Table 1). So, if relatively high [$UQ_{10}H_2$] (e.g., $> 4 \times 10^{-4}$ M as in $UQ_{10}D_2$) had been employed, the k_{obsd} value would have become very large (eq 9), and the [Toc•] would largely have decreased from the initial concentration (eq 7) within the time required to completely mix $UQ_{10}H_2$ with Toc• and to make the solution homogeneous. In order to evaluate k_{obsd} and k_{UQ} accurately, it is desirable to analyze the main decay profile of [Toc•] rather than the tail portion of the time-profile. Accordingly, we employed some relatively low [$UQ_{10}H_2$]'s in the evaluation of k_{UQ}^H, whose value thus obtained for α-Toc• at 25 °C was consistent with those reported previously.[17,19]

The k_{UQ} value of reaction 4 for α-Toc•, having three electron-donating methyl groups, is less than the corresponding value for β-Toc•, having two methyl groups (Table 1). When the electron-donating property of the substituent in Toc• is weak, the k_{UQ} value is large. This result suggests that an electron-transfer plays a role in reaction 4. A similar result was previously obtained for reaction 5.[37] An electron-transfer in reaction 1 was also suggested by the results of conventional single-mixing stopped-flow spectroscopy[39,43,44] and femtosecond spectroscopy[45] as well as of time-dependent density functional calculation.[46] It is interesting that β-TocH is more effective in antioxidant regeneration (reactions 4 and 5) than α-TocH, which is the most biologically active of natural TocH's.

Figure 7 shows Arrhenius plots of the k_{UQ}^H and k_{UQ}^D values, and linear relationships with negative slopes between log k_{UQ}^H (log k_{UQ}^D) and the reciprocal of the absolute temperature ($1/T$) can be seen in the plots, from which the activation energies

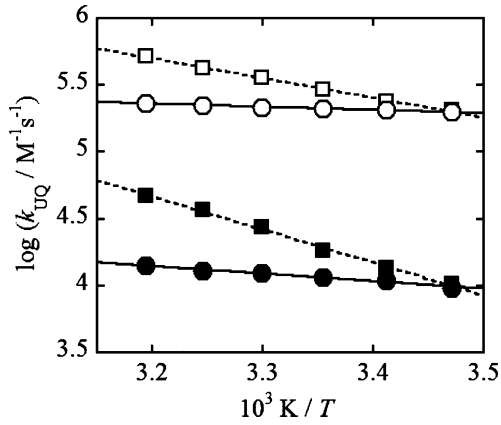

Figure 7. Arrhenius plots of k_{UQ}'s for reactions of $UQ_{10}H_2$ and α-Toc• in EtOH (open circles), of $UQ_{10}D_2$ and α-Toc• in EtOD (filled circles), of $UQ_{10}H_2$ and β-Toc• in EtOH (open squares), and of $UQ_{10}D_2$ and β-Toc• in EtOD (filled squares) (reaction 4).

(E^H and E^D) and the frequency factors (A^H and A^D) can be evaluated according to eq 10.

$$\log k_{UQ}^{H(D)} = -0.434E^{H(D)}/RT + \log A^{H(D)} \qquad (10)$$

where R denotes the gas constant. The ratio of k_{UQ}^H to k_{UQ}^D (k_{UQ}^H/k_{UQ}^D) at 25 °C, E^H, E^D, their difference ($E^D - E^H$), log A^H, and log A^D are listed in Table 2. In general, when the proton tunneling effect plays an important role, a substantial deuterium kinetic-isotope effect appears, and large values of k_{UQ}^H/k_{UQ}^D and $E^D - E^H$ are obtained.[47,48] Additionally, for reaction 4, a substantial deuterium kinetic-isotope effect on k_{UQ} (k_{UQ}^H and k_{UQ}^D) and the activation energy (E^H and E^D) is illustrated in Tables 1 and 2. The k_{UQ}^H/k_{UQ}^D values for α- and β-Toc•'s (18.3 and 15.9, respectively) exceed the maximum semiclassical ratio (6−8).[47,48] The $E^D - E^H$ values for α- and β-Toc•'s (6.1 and 18.6 kJ/mol, respectively) also exceed the maximum semiclassical difference (1.3−4.2 kJ/mol).[47,48] These results clearly show that the proton tunneling effect plays an important role in reaction 4 for Toc• originating from natural TocH in vitro as well as in reaction 4 for 5,7-diisopropyl-Toc•[38,39] and in various antioxidant reactions.[39,43,44,49,50] The proton tunneling was previously shown to also contribute to various vital functions such as mutation-suppression by vitamin C.[44,51−60] It is very interesting that the microscopic quantum-mechanical tunneling effect could manifest itself in macroscopic vital functions.

In connection with our experimental results, the computational study on the reaction between UQH_2 and phenoxyl radical (Ph-O•) by Yamamoto and Kato[61] is interesting. Since the moiety of phenoxyl radical contained in Toc• plays a key role in reaction 4, we can interpret our experimental results on the basis of their computational results. They thought that reaction 4 can proceed by proton tunneling below the transition state, and our experimental results for reaction 4 are supported by their computational results. In the later part of this paper, we will discuss our present and previous experimental results in conjunction with their computational results.

As mentioned in the introduction, the tunneling effect does not contribute to the regeneration reaction of natural TocH (α- and β-TocH's) by AsH^- (reaction 5),[37] judging from the fact that the ratio k_{VC}^H/k_{VC}^D corresponding to k_{UQ}^H/k_{UQ}^D (see reactions 5-H and 5-D) does not exceed the maximum semiclassical ratio (6−8).[47,48]

$$AsH^- + Toc\bullet \xrightarrow{k_{VC}^H} As^-\bullet + TocH \qquad (5\text{-H})$$

$$AsD^- + Toc\bullet \xrightarrow{k_{VC}^D} As^-\bullet + TocD \qquad (5\text{-D})$$

where AsD^- denotes a deuterated ascorbate monoanion. This previous result for reaction 5 contrasts sharply with the present result for reaction 4 in which the tunneling effect plays an important role. Thus, whether or not the tunneling effect plays an important role depends on the antioxidant reagent ($UQ_{10}H_2$ or AsH^-) employed in the regeneration reaction of natural TocH.

The reason for the contrast between reactions 4 and 5 for Toc• originating from natural TocH can be explained in the following way. First, let us examine the case in which reactions 4 and 5 proceed semiclassically. Since reactions 4-H and 5-H are transfers of protium (^1H), the possibility of proton tunneling is present. Accordingly, we will examine reactions 4-D and 5-D for α-Toc•; that is, we will compare the deuterium transfer from $UQ_{10}D_2$ to α-Toc• (reaction 4-D for α-Toc•) and the one from AsD^- (reaction 5-D for α-Toc•). Since the tunneling effect does not have a very large influence on the deuterium transfers compared with protium (^1H) transfers in a simple physical picture, the activation energies and rate constants in reactions 4-D and 5-D approximately reflect the real reaction potential-barriers and the rates of the semiclassical jumps over the barriers, respectively. In these deuterium transfers, the rate constant of reaction 5-D at 25 °C (4.87×10^5 M^{-1} s^{-1})[37] is much larger that of reaction 4-D (1.16×10^4 M^{-1} s^{-1}, Table 1), and the activation energy of reaction 5-D (6.4 kJ/mol)[37] is considerably less than that of reaction 4-D (10.6 kJ/mol, Table 2). Thus, when reactions 4 and 5 proceed semiclassically, reaction 5 is more favorable to the regeneration of natural TocH than reaction 4 under our experimental conditions.[62]

Next we will take the possibility of proton tunneling into account. Although reaction 5-D has a much lower potential-barrier than reaction 4-D as mentioned above,[62] the potential barrier of reaction 5-H for α-Toc• (4.0 kJ/mol) is still less than that of reaction 5-D (6.4 kJ/mol),[37] because the zero point energy of the OH vibration in AsH^- is larger than that of the OD vibration in AsD^-. Accordingly, reaction 5-H prefers to proceed by jumping semiclassically over the low potential barrier rather than to proceed by proton tunneling through the barrier (Figure 8a). As a result, the deuterium kinetic-isotope effect on k_{VC}^H/k_{VC}^D and the activation energy is small,[37] because both reactions 5-H and 5-D for α-Toc• proceed semiclassically.[47,48] By contrast, since the potential barrier of reaction 4 is high, the semiclassical jump over the barrier is difficult, and the proton tunneling takes place below the transition state in reaction 4-H (Figure 8b). As a result, a substantial deuterium kinetic-isotope effect on k_{UQ}^H/k_{UQ}^D and $E^D - E^H$ appears, because reactions 4-H and 4-D for α-Toc• proceed basically through different mechanisms (proton tunneling and semiclassical jump, respectively).[47,48] Also, in the above-mentioned computational results by Yamamoto and Kato,[61] the computational ratio corresponding to k_{UQ}^H/k_{UQ}^D decreases as the potential barrier decreases,[63] and their results are consistent with our above-mentioned view (Figure 8).

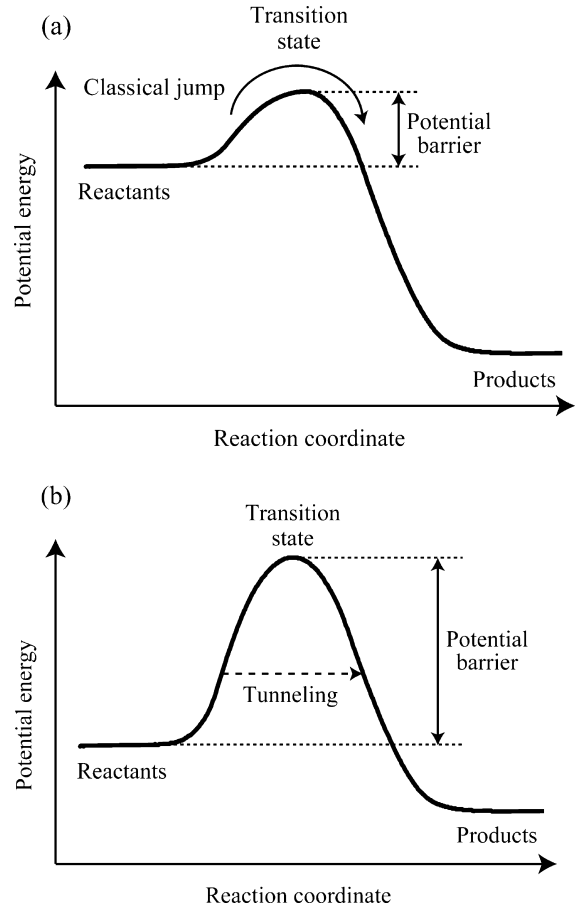

Figure 8. (a) Schematic potential-energy curve of reaction 5 for α- and β-Toc•'s. The reaction potential barrier is low. (b) Schematic potential-energy curve of reaction 4, whose barrier is high. The tunnel for protium (^1H) transfer is shown. This scheme is also applicable to reaction 5 for 5,7-diisopropyl-Toc•.

As mentioned in the preceding three paragraphs and the Introduction, the tunneling effect does not contribute to reaction 5 for α- and β-Toc•'s.[37] However, as mentioned in the Introduction, the tunneling effect plays an important role in reaction 5 for 5,7-diisopropyl-Toc•.[39] Thus, whether or not the tunneling effect contributes to reaction 5 depends on the structure of Toc•. Although the reason for the contrast between the reactions for these Toc•'s had not yet been elucidated, we can now explain it in terms of the difference in reaction potential barrier shown in Figure 8. When 5,7-diisopropyl-Toc• is used as Toc• in reaction 5, the steric hindrance due to the two isopropyl groups at the 5- and 7-positions prevents access of AsH^- to the O• at the 6-position of the Toc•. As a result, a high potential barrier appears in reaction 5 for 5,7-diisopropyl-

TABLE 2: k_{UQ}^H/k_{UQ}^D, E^H, E^D, $E^D - E^H$, log A^H, and log A^D Values of Reaction 4

reaction 4	k_{UQ}^H/k_{UQ}^D at 25 °C	E^H or E^D (kJ/mol)	$E^D - E^H$ (kJ/mol)	log A^H or log A^D [a]
$UH_{10}H_2$ + α-Toc•	18.3	4.5 ± 0.1	6.1	6.1
$UH_{10}D_2$ + α-Toc•		10.6 ± 0.7		5.9
$UH_{10}H_2$ + β-Toc•	15.9	27.9 ± 0.8	18.6	10.4
$UH_{10}D_2$ + β-Toc•		46.5 ± 1.4		12.6

[a] Because log A^H (log A^D) was obtained by extrapolating the linear log k_{UQ}^H (log k_{UQ}^D) versus $1/T$ plot in a limited $1/T$ range around room temperature to the intercept, these values have a large uncertainty.

Toc•, and so the proton tunneling plays an important role as shown in Figure 8b. In contrast, since such large steric hindrance is absent in α- and β-Toc•'s, the potential barrier of reaction 5 is low, and so the proton tunneling does not occur as shown in Figure 8a. Yamamoto and Kato also suggested in the above-mentioned paper[61] that the deuterium kinetic-isotope effect is not large in the absence of any steric hindrance preventing approach of two reactants, and their suggestion is consistent with our view. The reaction potential barrier of the regeneration of TocH would be higher in vivo than in a uniform solution, because the environment around the reactants in vivo prevents their approach. Accordingly, the proton tunneling could be induced in vivo according to the scheme given in Figure 8b.

On the basis of our experimental results mentioned above, we propose the following mechanism for reaction 3:

$$\begin{aligned} UQH_2 + Toc\bullet &\rightarrow [UQH_2 - - - Toc\bullet] \text{ (halfway to electron transfer)} \\ &\rightarrow [UQH\bullet - - - TocH] \text{ (proton tunneling)} \\ &\rightarrow [UQH\bullet - - - TocH] \end{aligned} \quad (11)$$

In the initial state of the reaction, UQH_2 and Toc• approach each other, and their electron clouds begin to overlap. Because UQH_2 and Toc• are respectively an electron-donor and an electron-acceptor, the transition state of reaction 3 has the property of an electron-transfer transition-state. In reality, however, when UQH_2 and Toc• approach each other to some extent ([UQH_2 --- Toc•]), the proton tunneling takes place below the transition state. The proton tunneling allows the reaction to proceed by cutting a corner on the potential energy surface. Finally, UQH• and TocH separate from each other. To avoid misleading, it should be noted that mechanism 11 does not refer to a stepwise pathway involving mechanically distinct electron transfer and proton tunneling but to a single chemical reaction involving electron transfer in concert with proton tunneling. The electron and proton move in one kinetic step, although, strictly speaking, the light electron begins to move before the heavy proton moves. The concerted reaction can proceed more preferentially than any stepwise reaction, because a stepwise reaction goes through an energetic intermediate.[64,65] Also, in the above-mentioned paper by Yamamoto and Kato,[61] the reaction of UQH_2 was interpreted as a proton-coupled electron transfer.[64,65] At the transition state, the calculated dipole moment reaches a maximum, and its orientation is reversed,[61] indicating that the transition state has the property of an electron-transfer transition state. Their result is also consistent with the above-mentioned experimental result that the k_{UQ} value is large when the electron-donating property of the substituent in Toc• is weak. They also thought that the proton tunneling took place under the transition state, as mentioned above.[61] Their computational results and interpretation thus support our mechanism 11.

Next we will critically examine our results and discussion. In a previous paragraph, we mentioned that reaction 5 has a much lower potential-barrier than reaction 4. However, E^H of reaction 4-H for α-Toc• (4.5 kJ/mol, Table 2) is similar to the potential barrier of reaction 5-H for α-Toc• (4.0 kJ/mol).[37] The reason for this is that E^H of reaction 4-H does not refer to the real potential barrier but to the energy difference between the tunnel and the reactants (Figure 8b).

The E^H (E^D) and log A^H (log A^D) values for β-Toc• are much larger than those of α-Toc• (Table 2). The reason for this would be the isokinetic relationship (compensation effect)[66,67] as pointed out in our previous paper.[37] In the isokinetic relationship, the correlation between the enthalpy and entropy changes in a series of related reactions results in E^H (E^D) being proportional to log A^H (log A^D).

Yamamoto and Kato mentioned, in the introduction of their paper,[61] an inverse deuterium kinetic-isotope effect (a slight decrease of k_{UQ}^H/k_{UQ}^D with a decrease in temperature) given in our previous paper on reaction 4 for 5,7-diisopropyl-Toc•.[38] However, the effect might originate from solvation as explained in our subsequent paper.[39]

As mentioned above, when reactions 4 and 5 proceed semiclassically, reaction 5 is more favorable to the regeneration of natural TocH than reaction 4 under our experimental conditions. Since the semiclassical routes on the potential-energy curves of reactions 4 and 5 are the electron transfer from the antioxidant reagent to Toc•, it is natural that reaction 5 for AsH^- having lower oxidation potential is semiclassically more favorable than reaction 4 for $UQ_{10}H_2$ having higher oxidation potential.[43] Then, the ratio of the rate constant of reaction 5[37] to that of reaction 4 (Table 1) is very large ($k_{VC}^D/k_{UQ}^D = 42.0$ for α-Toc• at 25 °C). However, when the proton tunneling plays a role, the corresponding ratio is reduced ($k_{VC}^H/k_{UQ}^H = 12.9$). Thus, the proton tunneling relatively accelerates reaction 4 compared with reaction 5, and the tunneling effect would give reaction 4 an advantage to the regeneration of TocH in vivo besides the advantages mentioned in the Introduction.

In our previous studies,[38,39] we used non-natural 5,7-diisopropyl-Toc• as Toc• in reactions 4 and 5, because $UQ_{10}H$•, As^-•, and natural Toc• are so short-lived that the examinations of the deuterium kinetic-isotope effects in reactions 4 and 5 through detection of these radicals were difficult with conventional single-mixing stopped-flow spectroscopy. The use of stable 5,7-diisopropyl-Toc• allowed us to study the deuterium kinetic-isotope effects with the conventional method. However, since the above-mentioned steric hindrance due to the two isopropyl groups prevents access of the antioxidant reagent to 5,7-diisopropyl-Toc•, its kinetics is often different from that for natural Toc• having little such steric hindrance. Accordingly, in our present and recent[37] works we have studied reactions 4 and 5 for α- and β-Toc•'s by using a double-mixing stopped-flow spectrophotometer, which has allowed us to examine the deuterium kinetic-isotope effect in the regeneration reaction of natural TocH through detection of the short-lived Toc•. Thus, the double-mixing stopped-flow spectrophotometer is a powerful tool in detailed studies of the regeneration reaction.

4. Conclusions

A kinetic study of reaction 4 was carried out by means of double-mixing stopped-flow spectroscopy. The proton tunneling effect plays an important role in reaction 3 that is advantageous to living organisms. When the reaction potential barrier is high and/or the steric hindrance prevents access of the antioxidant reagent to Toc•, the tunneling effect contributes to the regeneration of TocH.

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