Tunneling Effect in Antioxidant Reaction of Flavonoid

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A study of the kinetics of the proton-transfer reaction in flavonoid in ethanol solution by means of stopped-flow spectroscopy indicated that proton tunneling plays an important role in the antioxidant reaction.

Recent studies show that one of the causes of aging is lipid peroxyl radicals (LOO●'s) formed by the reactions of lipids and oxygen.[1] The living body, however, has a way to scavenge LOO● and thus help prevent aging: it is the so-called antioxidant reaction. In a previous paper,[2] some of us reported that proton tunneling plays an important role in the antioxidant reaction of vitamin E. However, although the living body includes various antioxidant molecules besides vitamin E, the tunneling effects in their antioxidant reactions have not yet been studied in detail.

In this communication, we report the tunneling effects in the antioxidant actions of some flavonoids. Flavonoids are natural polyphenolic compounds widely distributed in foods and plants and display pronounced biological activities, which protect against coronary heart disease, cancer, inflammation, etc.[3,4] A notable feature of flavonoids is their high reactivity toward active free radical species. Since flavonoids are localized near the surface of membranes, the reaction between flavonoids and LOO● is expected to take place there and dehydro-flavonoid radicals would be produced (Figure 1).[5,6] In this study, we measured the second-order rate constants (k_s's) for the reactions of three kinds of flavonoids (flavonol, morin, and fisetin, abbreviated as FlOH) with 2,6-di-*t*-butyl-4-(4-methoxyphenyl)-phenoxyl (ArO●) in ethanol solutions and examined their deuterium kinetic-isotope-effects.

$$\mathrm{ArO\bullet} + \mathrm{FlOH} \xrightarrow{k_s} \mathrm{ArOH} + \mathrm{FlO\bullet} \quad (1)$$

where FlO● is a dehydro-flavonoid radical. Since LOO● is not stable, we instead used stable ArO●. Reaction 1 is a proton-transfer reaction and is expected to show a significant deuterium kinetic-isotope-effect if proton tunneling takes place.[2,7]

The structures of the flavonoid molecules (flavonol, morin, and fisetin) studied in this work are shown in Figure 2.

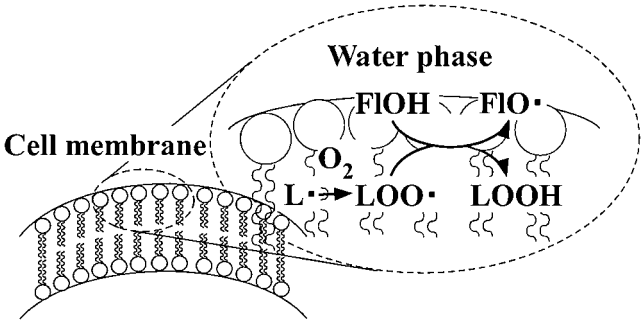

Figure 1. Scheme of LOO● production and a part of antioxidant reaction of FlOH at the interface of the cell membrane.

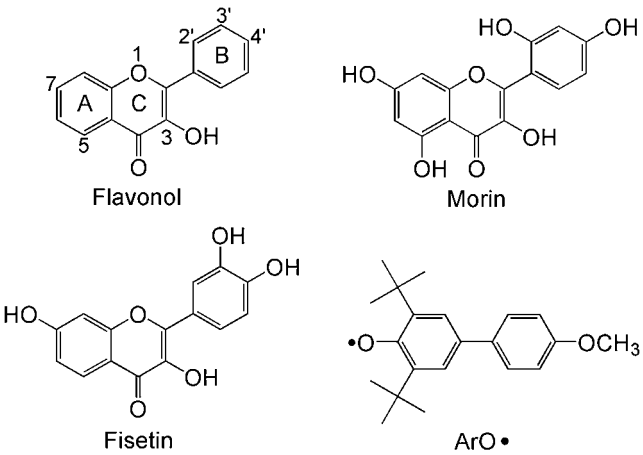

Figure 2. Structures of molecules used in the present work.

Commercially obtained ethanol (EtOH) was dried and purified by distillation. Flavonol, morin, fisetin, and ethanol-*d* (C_2H_5OD and EtOD) are commercially available and were used without purification. When FlOH is dissolved in EtOD, the hydrogen atom of the OH group is easily replaced by a deuteron to yield the deuterated molecule (FlOD). This replacement was confirmed by ^{1}H NMR spectra. The preparation of ArO● has been reported previously.[8]

The setup and experimental procedures for the measurement of k_s have been described in detail elsewhere.[2] Briefly, the kinetic data of reaction 1 were obtained with an Unisoku stopped-flow spectrometer model USP-500 or a Shimadzu UV–visible recording spectrophotometer (UV-2100S) equipped with a cell positioner CPS-260, by mixing equal volumes of EtOH (EtOD) solutions of FlOH (FlOD) and ArO● under a nitrogen atmosphere. Reaction 1 was studied under pseudo-first-order conditions ([FlOH (FlOD)] $\gg$ [ArO●]). [FlOH (FlOD)] and [ArO●] refer to the molar concentrations of FlOH (FlOD) and ArO●, respectively. Although ArO● was stable in the absence of FlOH (FlOD), when an EtOH (EtOD) solution with excess FlOH (FlOD) was added to the ArO● solution, the ArO● absorption peak disappeared immediately. The absorption decay of ArO● at 580 nm was well-characterized by a single-exponential decay. Figure 3 shows the change in absorption spectrum during reaction 1 for morin. The pseudo-first-order rate constant (k_{obsd}) was determined by evaluating the decrease in the absorbance of ArO●. As shown in Figure 4,

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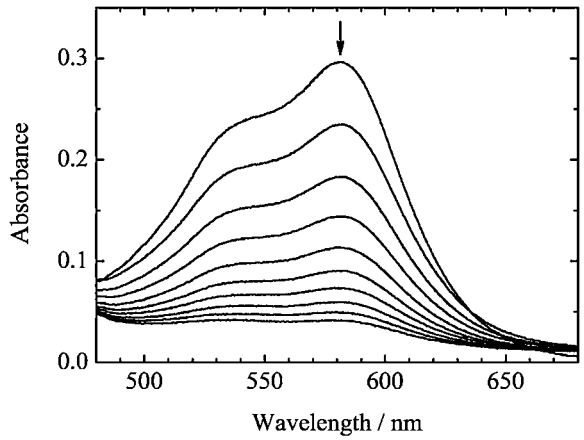

Figure 3. Change in absorption spectrum during reaction 1 for morin at 25 °C. The arrow indicates the decrease in the absorbance of ArO•. 10 s intervals with $[\text{Morin}]_{t=0} = 1.52 \times 10^{-3}$ M and $[\text{ArO}\bullet]_{t=0} = \approx 7.0 \times 10^{-5}$ M.

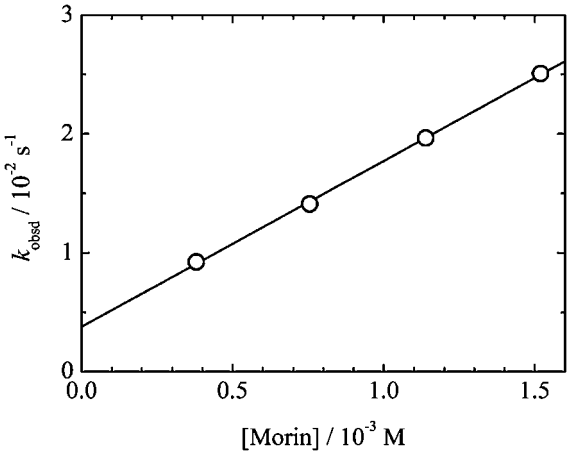

Figure 4. Dependence of k_{obsd} on [Morin] in the reaction of morin and ArO• in EtOH.

k_{obsd} was linearly dependent on [FlOH (FlOD)]. The rate equation is thus expressed as

$$-\text{d[ArO}\bullet\text{]}/\text{d}t = k_{obsd}[\text{ArO}\bullet] = \{k_0 + k_s[\text{FlOH (FlOD)}]\}[\text{ArO}\bullet] \quad (2)$$

where k_0 is the rate constant for natural decay of ArO• under our measurement conditions. The k_s value was obtained by plotting k_{obsd} against [FlOH (FlOD)] (Figure 4).

The k_s values in ethanol solution are 14.4, 5.72×10^{-2}, and 1.24×10^2 M^{-1} s^{-1} for morin, flavonol, and fisetin, respectively (Table 1). The structure–activity relationship of ArO• scavenging reaction by flavonoids has been discussed in a previous report.[3] Briefly, the ArO• scavenging activity of 1) the –OH groups at the A-ring is very weak and almost negligible, 2) the –OH groups at the B-ring is high (that is, the existence of a catechol structure, which has 3′-OH and 4′-OH, is especially important), and 3) the 3-OH group at the C-ring is very weak. Since the k_s values of the reaction with ArO• for apigenin and naringenin were very small, it was difficult to estimate their deuterium kinetic-isotope-effects. The estimation for quercetin was also difficult since the absorption peak of dehydro-quercetin radical overlapped with that of ArO•.[3]

Table 1. k_s at 25 °C, k_s^H/k_s^D at 25 °C, E_{act}, and $E_{act}^D - E_{act}^H$

Molecule	k_s at 25 °C /M^{-1} s^{-1}	k_s^H/k_s^D	E_{act} /kJ mol^{-1}	$E_{act}^D - E_{act}^H$ /kJ mol^{-1}
Morin	14.4	14.5	30.0 ± 0.5	12.3
Morin-d_5	9.90×10^{-1}		42.3 ± 0.5	
Flavonol	5.72×10^{-2}	6.8	37.9 ± 3.3	15.6
Flavonol-d	8.41×10^{-3}		53.5 ± 3.3	
Fisetin	1.24×10^2	8.3	19.2 ± 1.4	22.3
Fisetin-d_4	14.9		41.5 ± 1.4	
γ-Tocopherol[a)]	2.73×10^3 [b)]	14.6[c)]	20.9 ± 2.5	8.0
γ-Tocopherol-d[a)]	1.99×10^2 [b)]		28.9 ± 1.7	

a) Ref. 2. b) See Figure 6 of Ref. 2. c) Average ratio at 15–35 °C.

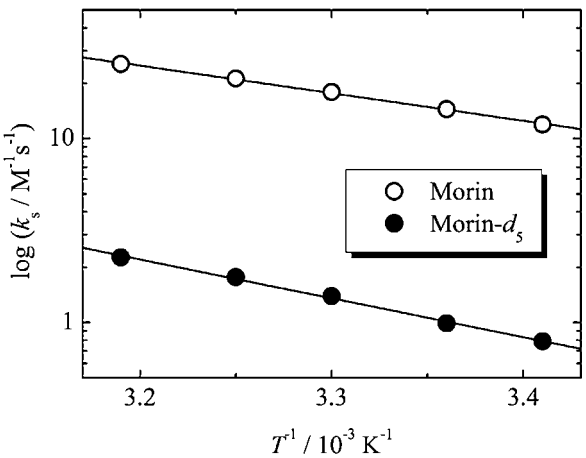

Figure 5. Arrhenius plot of k_s for morin (morin-d_5) with ArO• in EtOH (EtOD).

Figure 5 shows the Arrhenius plots of the k_s values of morin and morin-d_5 (k_s^H and k_s^D), indicating linear relationships between log k_s and $1/T$. The k_s value at 25 °C, the ratio of k_s^H to k_s^D (k_s^H/k_s^D) at 25 °C, and the activation energy (E_{act}) are listed in Table 1 together with those of γ-tocopherol,[2] which is the major form of vitamin E in the United States diet[9] and has important antioxidant and other properties.[10] In Table 1, substantial deuterium kinetic-isotope-effects on k_s and E_{act} are summarized. The k_s^H/k_s^D value (14.5) of morin exceeds the maximum semiclassical ratio (6–8)[7] and is close to that of γ-tocopherol (14.6). The E_{act} difference ($E_{act}^D - E_{act}^H$) between morin and morin-d_5 (12.3 kJ mol^{-1}) also exceeds the maximum semiclassical difference (1.3–4.2 kJ mol^{-1})[7] as well as that of γ-tocopherol (8.0 kJ mol^{-1}). These results clearly show that the tunneling effect plays an important role in the reaction 1 of morin in vitro as well as of γ-tocopherol. Similar deuterium kinetic-isotope-effects were also obtained for flavonol and fisetin (Table 1) though the k_s^H/k_s^D value of flavonol is less than anticipated. We here suggest that the deuterium kinetic-isotope-effects mainly originate from the 3-OH on the C-ring for flavonol, the 2′, 4′-OH on the B-ring for morin, and the 3′, 4′-OH on the B-ring for fisetin.

The E_{act} values of fisetin and fisetin-d_4 showed a large deuterium-isotope-effect though the k_s^H/k_s^D value was small. We also obtained a similar result for the reaction of β-tocopheroxyl radical and sodium ascorbate previously.[11] Since

the structure of fisetin is not similar to that of sodium ascorbate, the phenomenon may not come from the structure of fisetin. At present we do not have an unambiguous explanation for these phenomena, but a possible reason is an isokinetic relationship (compensation effect).[11–13]

From the present results, one might expect that the tunneling effect contributes to the inhibition of lipid peroxidation by FlOH in vivo. It is very interesting that the microscopic quantum-mechanical tunneling effect could manifest in a macroscopic vital function. Proton tunneling has previously been shown to contribute also to other vital functions.[2,14–17]

In summary, the present study carried out by means of stopped-flow spectroscopy has shown that proton tunneling of FlOH plays an important role in an antioxidant reaction that is advantageous in vivo.

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