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A large kinetic isotope effect in the reaction of ascorbic acid with 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO•) in aqueous buffer solutions†

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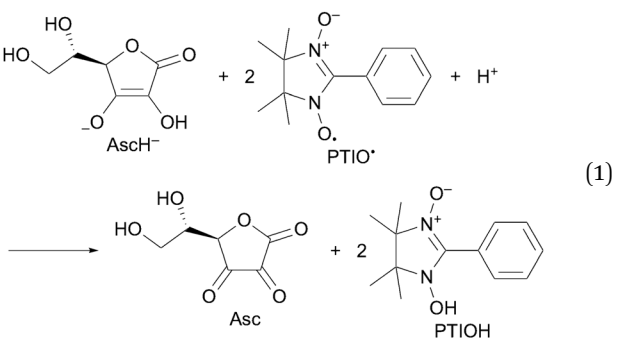
A large kinetic isotope effect (KIE, k_H/k_D) of 12.8 was observed for the hydrogen-transfer reaction from ascorbic acid to 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO•) in a phosphate buffer solution (0.05 M, pH/pD 7.0) at 298 K. The isotopic difference in the activation energies (6.8 kJ mol^{-1}) determined from the temperature dependence of the KIE suggests that quantum mechanical tunneling may partly play a role in the reaction, although the isotopic ratio of the Arrhenius prefactor (A_H/A_D = 0.86) is within the semiclassical limits.

Quantum mechanical tunneling in hydrogen-transfer reactions[1–3] in biological redox systems has attracted considerable attention with regard to the quantum mechanical behaviour in biology in recent years.[4] Uršić *et al.* reported that a large kinetic isotope effect (KIE, k_H/k_D) of 24.2 was observed in water for the hydrogen-transfer reaction from ascorbic acid ($AscH_2$), one of the representative water-soluble antioxidants, to 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) radicals.[5] This is the first report about hydrogen tunneling in a reaction involving $AscH_2$. On the other hand, Li has recently reported a new and simple antioxidant assay *in vitro* using 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide radicals (PTIO•), one of the nitronyl nitroxide radicals, where a hydrogen transfer occurred from antioxidants to PTIO•.[6] However, little is known about the kinetics of the reaction between antioxidants and PTIO•, as well as the KIE. We report herein the observation of a large kinetic isotope effect for the reaction of $AscH_2$ with PTIO• in a phosphate buffer. A possibility of the involvement of quantum mechanical tunneling is also discussed based on the temperature dependence of the KIE.

When $AscH_2$ was added to the phosphate buffer solution (0.05 M, pH 7.0) of PTIO•, the bands at 345 and 560 nm decreased immediately with clear isosbestic points at 218, 244, 279 and 313 nm as shown in Fig. 1. Since the pK_a value of $AscH_2$ is reported to be 4.1,[7] $AscH_2$ undergoes deprotonation and exists in its anionic form, $AscH^-$, in phosphate buffer solution (0.05 M, pH 7.0). Thus, this spectral change indicates that $AscH^-$ efficiently scavenged PTIO• in phosphate buffer. The spectral titration (inset of Fig. 1) shows that the stoichiometry of the reaction is given by eqn (1), where $AscH^-$ reacts with 2PTIO•.



(1)

The decay of the absorbance at 560 nm monitored by a stopped-flow technique obeyed pseudo-first-order kinetics, when the concentration of $AscH_2$ ([$AscH_2$]) was maintained at more than a 10-fold excess of PTIO• concentration (Fig. 2). The pseudo-first-order rate constants (k_{obs}) linearly increased with increasing [$AscH_2$] (Fig. 3). From the slope of the linear plot, the second-order rate constant (k_H) for the scavenging reaction of PTIO• by $AscH_2$ [eqn (2)] was determined in a phosphate buffer

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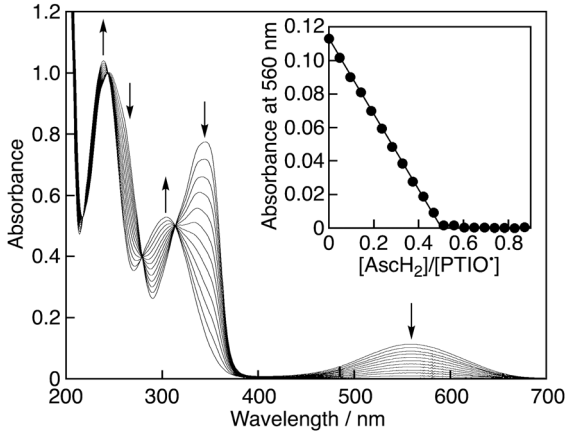
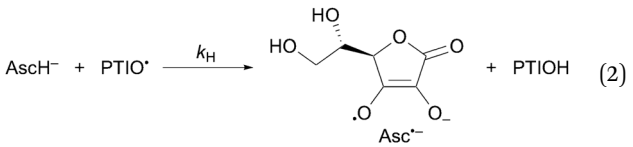


Fig. 1 Spectral change observed upon addition of $AscH_2$ (0–5.2 × 10^{-5} M, 4.6 × 10^{-6} M each) to PTIO$^{\bullet}$ (1.0 × 10^{-4} M) in phosphate buffer (0.05 M, pH 7.0). Inset: Plot of the absorbance at 560 nm *vs.* [$AscH_2$]/[PTIO$^{\bullet}$].

(0.05 M, pH 7.0) to be 2.4 × 10^3 M^{-1} s^{-1}. This value is smaller than that determined for the reaction between $AscH_2$ and β-cyclodextrin-solubilised 2,2-diphenyl-1-picrylhydrazyl radicals (DPPH$^{\bullet}$)[8] under the same experimental conditions (k_H = 5.6 × 10^3 M^{-1} s^{-1}). Thus, the reactivity of PTIO$^{\bullet}$ toward $AscH_2$ is lower than that of DPPH$^{\bullet}$.



When H_2O was replaced by D_2O to prepare the phosphate buffer, the exchangeable O–H protons in $AscH_2$ are replaced by deuterons from D_2O to produce $AscD_2$. The second-order rate constant (k_D) thus determined for the reaction of $AscD_2$ with PTIO$^{\bullet}$ was significantly decreased to be 1.9 × 10^2 M^{-1} s^{-1}.

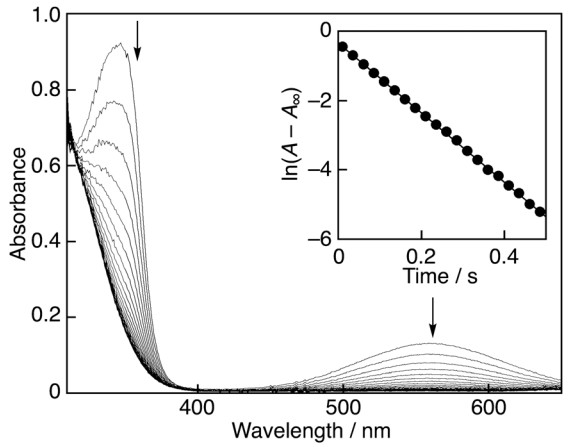


Fig. 2 Spectral change (interval: 25 ms) observed during the reaction of $AscH_2$ (4.0 × 10^{-3} M) with PTIO$^{\bullet}$ (9.4 × 10^{-5} M) in phosphate buffer (0.05 M, pH 7.0) at 298 K. Inset: The first-order plot of the absorbance at 560 nm.

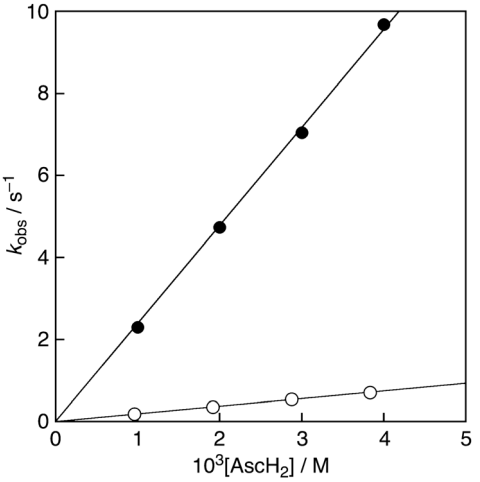


Fig. 3 Plots of pseudo-first-order rate constants (k_{obs}) *vs.* concentrations of $AscH_2$ in phosphate buffer (H_2O, 0.05 M, pH 7.0) (closed circles) and in phosphate buffer (D_2O, 0.05 M, pD 7.0) (open circles).

Thus, the KIE (k_H/k_D) is calculated to be 12.8. Such a large KIE value has clearly precluded an electron-transfer pathway in the oxidation reaction of $AscH_2$ by PTIO$^{\bullet}$. This value is beyond the maximum expected semiclassical value of 7.9 for the dissociation of the O–H bond.[9] We also performed the reaction of $AscH_2$ with PTIO$^{\bullet}$ in the temperature range from 288 to 308 K and the k_H and k_D values were determined from the slopes of the linear plots of the k_{obs} values *vs.* concentrations of $AscH_2$ or $AscD_2$ (Table 1).

Furthermore, as the Arrhenius plots are shown in Fig. 4, linear correlations of ln k_H *vs.* T^{-1} and ln k_D *vs.* T^{-1} were observed in the reaction of $AscH_2$ with PTIO$^{\bullet}$ in the whole temperature range. From the intercepts of Fig. 4, the isotopic ratio of the Arrhenius prefactor (A_H/A_D = 0.86) was obtained. This value can be fitted within the semiclassical limits of 0.7–1.4 for the A_H/A_D value in a hydrogen-transfer process.[9] The isotopic difference in the activation energies E_a(D)–E_a(H) was 6.8 kJ mol^{-1}, which is beyond the difference in zero-point energies of 5.1 kJ mol^{-1}.[9] A large A_H/A_D value ($\gg$1) in hydrogen transfer of some enzymes has been reported by Klinman *et al.*, proposing a full tunneling mode to explain such observation.[3] Furthermore, Uršić *et al.* claimed that quantum mechanical tunneling plays a role in the reaction between $AscH_2$ and TEMPO in water–dioxane (1 : 1 v/v) based on the large KIE value of 31.1 and E_a(D)–E_a(H) value of 8.2 kJ mol^{-1}, although the A_H/A_D value is 1.2.[5] Thus, quantum mechanical tunneling may

Table 1 k_H, k_D and k_H/k_D values for the reaction of $AscH_2$ or $AscD_2$ in phosphate buffer solutions (0.05 M, pH 7.0 or pD 7.0)

T/K	k_H/M^{-1} s^{-1}	k_D/M^{-1} s^{-1}	k_H/k_D
288	1.5 × 10^3	9.6 × 10	15.6
293	2.0 × 10^3	1.5 × 10^2	13.3
298	2.4 × 10^3	1.9 × 10^2	12.8
300	2.8 × 10^3	1.9 × 10^2	14.8
303	2.9 × 10^3	2.2 × 10^2	13.3
308	3.1 × 10^3	2.6 × 10^2	12.2

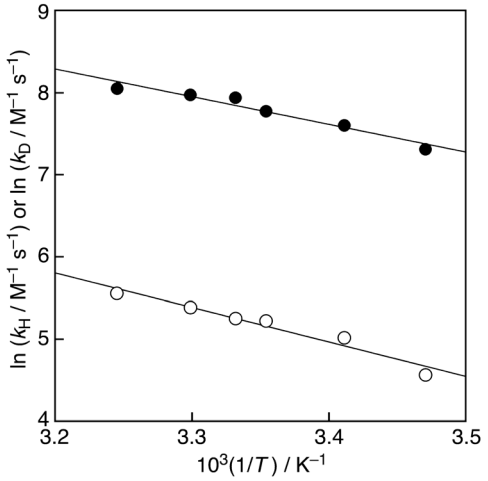


Fig. 4 Arrhenius plots of ln k_H *vs.* T^{-1} (closed circles) and ln k_D *vs.* T^{-1} (open circles) in phosphate buffer (H_2O, 0.05 M, pH 7.0) and in phosphate buffer (D_2O, 0.05 M, pD 7.0), respectively.

partly play a role in the reaction between $AscH_2$ and PTIO$^{\bullet}$ in a phosphate buffer solution.

In summary, a large KIE was observed for the hydrogen-transfer reaction from $AscH_2$ to PTIO$^{\bullet}$. The temperature dependence of the KIE suggests that quantum mechanical tunneling may partly play a role in the reaction, although the isotopic ratio of the Arrhenius prefactor (A_H/A_D) is within the semiclassical limits. Because there are only a few reports about a large KIE in a reaction involving $AscH_2$, this study provides valuable information for the biological redox reactions including ascorbic acid.

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Conflicts of interest

There are no conflicts to declare.

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