Kinetic Investigation of Reaction of Ascorbate and Hydroxyl Radical Adduct of DMPO (5,5-Dimethyl-1-pyrroline *N*-Oxide) Studied by Stopped-flow ESR

Yasuhiro Sakurai, Hodaka Sanuki, Rushiru Komatsu-Watanabe, Tomoko Ideguchi, Naoki Yanagi, Kiyoshi Kawai, Kenji Kanaori, and Kunihiko Tajima*

Department of Biomolecular Engineering, Kyoto Institute of Technology, Matsugasaki, Sakyo-ku, Kyoto 606-8585

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By means of a stopped-flow electron spin resonance (ESR) method, the second-order rate constant, defined for reduction of the hydroxyl radical adduct of 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO/OH) by ascorbic acid at pH 7.4, was evaluated to be $5.2 \pm 0.2 \times 10^3\,M^{-1}\,s^{-1}$, which was at least 700 times faster than that of 2,2,6,6-tetramethyl-4-hydroxylpiperidine-*N*-oxyl (TEMPOL) radical ($7\,M^{-1}\,s^{-1}$).

In biochemical and chemical reaction systems, several short-lived free radicals have been detected by spin-trapping ESR technique using spin-trapping reagents, which react with the radicals to form relatively stable nitroxyl radicals.[1] 5,5-Dimethyl-1-pyrroline *N*-oxide (DMPO) is a widely used spin-trapping reagent, which can trap carbon- and oxygen-centered radicals, such as hydroxyl and superoxide anion radicals, so called reactive oxygen species (ROS).[2] The DMPO spin-trapping technique has frequently been applied for detection of ROS formed in living cells or tissues.[3] In biological systems, the spin adducts of DMPO are readily depleted to diamagnetic substances. For example, the superoxide anion radical remarkably accelerates the decomposition of its DMPO adduct (DMPO/OOH, $4.9 \times 10^6\,M^{-1}\,s^{-1}$).[4] In addition, ascorbic acid has been believed to be a powerful reducing reagent for nitroxyl radicals.[5] So far, however, little is known about the mechanisms of the reaction between DMPO spin adducts and ascorbic acid. In the present work, kinetic studies on the reaction between the hydroxyl radical adduct of DMPO (DMPO/OH) and ascorbic acid were conducted by means of a stopped-flow ESR system.[6]

The stopped-flow ESR spectra were recorded with a modified TE-100 (JEOL), combined with a micro-4-jet-mixer, a quartz capillary ESR cell (i.d. 0.86 mm, Wilmad), and a nitrogen gas drive syringe pump (JEOL), which was capable of mixing 0.2 mL of two reaction solutions within 50 ms. The optical absorption spectra were recorded with a photodiode-array spectrometer (Photal, MCPD 3000) coupled with a quartz optical cell (optical path-length 2.0 mm) connected to the mixer. DMPO (Labotec), TEMPOL (Sigma Aldrich), and ascorbic acid (Wako Pure Chemical) were used without purification. QII grade water (Millipore) was used for preparation of buffer solution (0.1 M, $M = mol/dm^3$, NaH_2PO_4–Na_2HPO_4) at pH 7.4, where ascorbic acid largely exists as ascorbate anion because its pK_a is 4.25 and 11.57.[7] To prevent autoxidation of ascorbate, the solution of ascorbic acid was prepared under argon condition and stored at 273 K.[8]

Stopped-flow optical measurements were made by mixing solutions of DMPO (2.0 mM) and ascorbate (100 μM) prepared at pH 7.4 under argon condition. After about 1.0 s from the mixing, the reaction solution displayed two absorption maxima at 228 and 265 nm (data not shown) derived from DMPO[4] and ascorbate,[9] respectively. The optical spectrum was monitored for 10 min at 10 s intervals; however, no significant spectral change was recognized, suggesting that the oxidative consumption of ascorbate by reaction with DMPO could be negligible in the present condition.

With reference to reports of Makino et al.[10] and Samuni et al.[11] the solution of DMPO/OH was prepared by sonication of aqueous DMPO. The sonolysis of argon-saturated DMPO solution (pH 7.4, 2.0 mM) was performed at 313 K for 10 min, by using a N-100L ultrasound cleaner (Verno) operating with 40 kHz oscillation frequency and 100 W output power. The resulting solution was immediately cooled to 273 K and stored for subsequent use. Stopped-flow ESR measurements were performed for the sonicated DMPO solution, after mixing with equal quantities (0.2 mL) of the buffer solution.[12] As depicted in Figure 1a, the resulting solution showed an ESR signal due to DMPO/OH ($g = 2.0046$, $a^N = a^H = 1.49$ mT).[2] The ESR intensity of DMPO/OH was unchanged over about 10 min at 298 K. After duplicate integration made for the ESR signal, the concentration of DMPO/OH after mixing (defined as $[DMPO/OH]_0$) was estimated to be 3.1 ± 0.1 μM by using TEMPOL as a primary standard.

Similar ESR measurements were continued for the same solution of DMPO/OH by mixing with a solution of ascorbate, where the concentration of ascorbate just after mixing (defined as $[ascorbate]_0$) was adjusted to 100 μM. As shown in Figure 1b, ESR spectrum recorded at 5 s after mixing revealed formation of a weak doublet signal ($g = 2.005$, $a^H = 0.18$ mT) ascribed to be the anion radical of ascorbic acid ($Asc^{-\bullet}$).[13] On

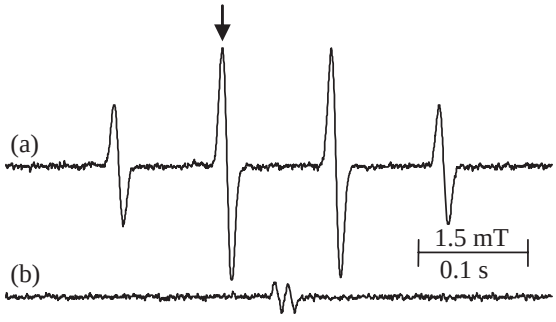

Figure 1. Stopped-flow ESR spectra recorded by field-sweep mode. (a) ESR spectrum recorded by mixing the sonicated DMPO solution (0.2 mL) and buffer solution (0.2 mL), (b) ESR spectrum recorded at 5 s after mixing solutions of DMPO/OH (3.1 μM, 0.2 mL) and ascorbate (100 μM, 0.2 mL). The concentration of DMPO/OH and ascorbate correspond to the initial concentration just after mixing. The downward arrow indicates the static magnetic field for the stopped-flow ESR measurements by time-sweep mode.

the other hand, the ESR signal due to DMPO/OH almost disappeared, indicating that the reaction of DMPO/OH and ascorbate resulted in formation of $Asc^{-\bullet}$ and a reduced form of DMPO/OH (DMPOH/OH), as formulated in eq 1.[14]

$$\text{DMPO/OH} + \text{ascorbate} \rightarrow \text{DMPOH/OH} + \text{Asc}^{-\bullet} \quad (1)$$

Stopped-flow ESR measurements by time-sweep mode were performed for the same solution of DMPO/OH by monitoring the decrease in the signal intensity of the second low-field line of DMPO/OH (Figure 1a). When the solution of ascorbate (200 μM, 0.2 mL) was mixed with DMPO/OH (6.2 μM, 0.2 mL), the concentration of the radical rapidly decreased and completely disappeared within about 8 s, as depicted in Figure 2. Analogous decay curves were also observed by changing $[\text{ascorbate}]_0$ from 100 to 25 μM, and the half-life period of the radical ($\tau_{1/2}$) showed stepwise increase depending on the decrease of $[\text{ascorbate}]_0$ (Figure 2). The $\tau_{1/2}$ values were very sensitive to $[\text{ascorbate}]_0$, so that strict deoxygenation was required for the reaction solution of ascorbate to obtain reliable kinetic parameters.

The line shape of observed decay curves exhibited single-exponential decay (Figure 2). Then the rate equation is expressed as eq 2. Based on the time dependence of the concentration of DMPO/OH, the pseudo-first-order rate constants (k_{obsd}) were estimated to be 0.52 ± 0.03, 0.38 ± 0.02, 0.28 ± 0.03, and $0.15 \pm 0.02\ s^{-1}$, respectively to $[\text{ascorbate}]_0$ at 100, 75, 50, and 25 μM.

$$-\text{d[DMPO/OH]}/\text{d}t = k_{obsd}[\text{DMPO/OH}] = k_2[\text{DMPO/OH}][\text{ascorbate}] \quad (2)$$

As evident from the inset of Figure 2, the plots of k_{obsd} vs. $[\text{ascorbate}]_0$ gave a straight line that coincides with the origin. In terms of least square treatment, the slope of the line was evaluated to be $5.3 \pm 0.1 \times 10^3\ M^{-1}\ s^{-1}$, where the correlation coefficient was estimated to be 0.983. Therefore, the second order rate constant (k_2, eq 2), defined for the reduction of DMPO/OH by ascorbate (eq 1), is determined to be $5.2 \pm 0.2 \times 10^3\ M^{-1}\ s^{-1}$, as a mean value of three independent measurements. The estimated k_2 value is at least 700 times larger than that reported for TEMPOL ($k_2 = 7\ M^{-1}\ s^{-1}$).[15] The present result provides experimental evidence that DMPO/OH is one of the most fragile nitroxyl radical to ascorbate reduction.

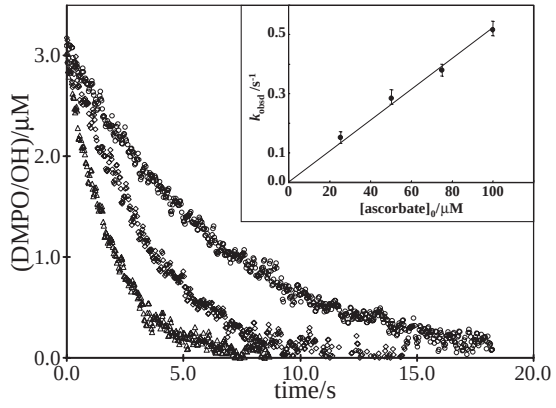

Figure 2. Stopped-flow ESR recorded for DMPO/OH by time-sweep mode, after mixing argon saturated solutions of DMPO/OH (3.1 μM, 0.2 mL) and of ascorbate (0.2 mL) with different concentration; 100 (△), 50 (◇), and 25 μM (○). The concentration of DMPO/OH and ascorbate correspond to the initial concentration just after mixing. Inset: Dependence of k_{obsd} on $[\text{ascorbate}]_0$ in aqueous solution at pH 7.4.

In the presence of ascorbic acid, the spin-trapping reaction occurring between DMPO and hydroxyl radical consisted of the following three reactions with different rate constants; (1) reaction of hydroxyl radical and DMPO ($2.8 \times 10^9\ M^{-1}\ s^{-1}$),[16] (2) reaction of hydroxyl radical and ascorbate ($1.1 \times 10^{10}\ M^{-1}\ s^{-1}$),[17] and (3) reduction of DMPO/OH by ascorbate ($5.2 \times 10^3\ M^{-1}\ s^{-1}$). By comparison of these bimolecular rate constants, we find that ascorbate affected much the concentration and the life-time of DMPO/OH, especially in the early reaction period of spin-trapping ESR measurements. Consequently, it seems to be difficult to achieve a steady-state concentration of DMPO/OH above the ESR detection limit. In many cases, a failure to detect ESR signal of DMPO/OH serves to be evidence for exclusion of hydroxyl radical formation in the system. It is necessary to confirm the existence of ascorbic acid in order to obtain correct conclusions regarding hydroxyl radical production based on the results of DMPO spin-trapping ESR. Further kinetic study for estimation of the k_2 values of DMPO derivatives are now in progress.

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