Carbon- and Nitrogen-Centered Radicals Produced from *L*-Lysine by Radiation-Induced Oxidation: A Pulse Radiolysis Study

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ABSTRACT

Radical species generated from the reactions of a basic amino acid, *L*-Lysine (Lys), with hydroxyl radicals ($^{\bullet}OH$) and sulfate radical anion ($SO_4^{-\bullet}$) have been detected by the method of pulse radiolysis. On the basis of electron transfer reactivities toward tetranitromethane (TNM), it was demonstrated that reducing carbon-centered radicals are generated as a result of hydrogen abstraction from CH_2 of Lys with a *G*-value of 1.9×10^{-7} mol J^{-1}. On the other hand, direct oxidation of *L*-Lys by $SO_4^{-\bullet}$ formed a transient species with different spectroscopic properties, most likely, the *ε-N*-centered Lys radical.

1. INTRODUCTION

Under aerobic conditions, amino acid residues of proteins are susceptible to attack by reactive oxygen species (ROS), and the damages in proteins and peptides are implicated in a variety of disease states, as well as in the progression of aging.[1-4] Varieties of amino acid radicals produced by ionizing radiations have been characterized by the methods of electron spin resonance (ESR) [5-10] and pulse radiolysis [11-14] in the last few decades.

Hydroxyl radical ($^{\bullet}$OH) generated under oxidative stress is an oxidizing radical species toward biomolecules, and is considered to induce harmful effects on living things. It has been reported that reaction of such primary radicals with nucleohistone in aqueous solution produces crosslinks between DNA bases and amino acids such as lysine (Lys) and arginine (Arg).[15,16] Since $^{\bullet}$OH induces oxidation of amino acids near the interfaces of proteins and solvent water molecules, radiation-induced footprinting coupled with mass spectrometry has become a powerful technique for mapping the solvent accessible surface of proteins.[17]

In the case of simple α-amino acid of glycine (Gly), $^{\bullet}$OH abstracts hydrogen from the carbon skeleton at neutral or acidic pH. In the basic pH range, $^{\bullet}$OH also directly attacks the nitrogen atom of the deprotonated amino group and initiate decarboxylation leading to the formation of a strongly reducing α-amino carbon-centered radical.[13,18,19]

Most spectroscopic studies have focused on the radical reactions of simple α-amino acids induced by $^{\bullet}$OH, on the other hand, limited studies have been reported for basic amino acids, such as Arg and Lys. Previous ESR spin trapping studies on $^{\bullet}$OH reaction with Lys identified *C*-centered radical structures generated as a result of $^{\bullet}$OH attack at the side chain. *C*-Centered radicals thus generated form DNA-protein crosslinks via addition to thymine base or radical-radical recombination with thymine radicals.[15,16] Recent product analysis studies on DNA-protein crosslink identified *N*ε-(guaninyl)-lysine adducts by photosensitized or chemical oxidation of guanine base in the presence of Lys derivatives.[21-23] Burrows and co-workers have demonstrated that attack of the ε-*N*-centered radical

(cation) generated by direct oxidation of Lys toward guanine base is involved in the crosslink formation.[22]

Here we describe the dynamic behavior of intermediate radicals produced in the reaction of *L*-Lys and oxidizing species such as $^{\bullet}OH$ and sulfate radical anion ($SO_4^{-\bullet}$) as investigated by the method of pulse radiolysis to understand the oxidative protein damage mechanism. Redox reactive radicals generated during the radiolysis were quantitated by the redox titration technique, by which we can detect oxidizing or reducing radicals of amino acids by converting the radicals into easily observable radical cation of *N,N,N',N'*-tetramethyl-*p*-phenylenediamine ($TMPD^{+\bullet}$) or nitroform anion (NF^-).[23,24]

2. EXPERIMENTAL SECTION

2.1 Materials

L-Lysine and *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD) were purchased from Wako Pure Chemical Industries. Potassium peroxodisulphate ($K_2S_2O_8$) and 2-methyl-2-propanol (*t*-BuOH) were purchased from Nacalai Tesque. Tetranitromethane (TNM) and *L*-norleucine were purchased from Aldrich Chemical. All chemicals were used as received.

2.2 Pulse radiolysis

Amino acids were dissolved in phosphate buffer solution (5.0×10^{-3} mol dm^{-3}) with water purified by a Millipore Milli-Q system. Radiolysis of water produces primary radical species including $^{\bullet}OH$, hydrated electron (e_{aq}^-), and hydrogen atom ($^{\bullet}H$) with *G*-values[25] of 2.9×10^{-7} mol J^{-1}, 2.9×10^{-7} mol J^{-1}, and 0.6×10^{-7} mol J^{-1}, respectively.

$$H_2O \rightarrow {}^{\bullet}OH,\ e^-_{aq},\ {}^{\bullet}H$$

For the $^{\bullet}OH$ reaction, the amino acid solutions were saturated with N_2O to scavenge e_{aq}^-.

$$e_{aq}^- + N_2O \rightarrow N_2 + {}^{\bullet}OH + OH^-$$

On the other hand, for the $SO_4^{-\bullet}$ reaction, aqueous solution of *L*-Lys solution containing $K_2S_2O_8$ (1.0×10^{-2} mol dm^{-3}) and *t*-BuOH (0.10 mol dm^{-3}) were saturated with Ar prior to the irradiation. Under the conditions, $^{\bullet}OH$ and e_{aq}^- are scavenged by *t*-BuOH and $S_2O_8^{2-}$, respectively.

$$^{\bullet}OH + t\text{-BuOH} \rightarrow t\text{-BuOH}(-H^{\bullet}) + H_2O$$

$$e_{aq}^{-} + S_2O_8^{2-} \rightarrow SO_4^{-\bullet} + SO_4^{2-}$$

The electron beam (7 Gy pulse^{-1}) was produced in a linear accelerator (High Voltage Engineering Co., Ltd.) giving 10-MeV electrons with a variable pulse width up to 5 μs; its peak current being about 0.4 A. The electron beam, spread by an aluminum plate 4 mm thick, entered into a quartz cell (light path length = 1.5 cm) filled with the sample solution through a brass slit and fell on a brass collector. The current was monitored as voltage on a condenser with a digital voltmeter having a holding mechanism. The analysis light emitted from a 300-W Xe lamp (L2479; Hamamatsu Photonics Co., Ltd.) was passed the cell perpendicularly to the electron pulses. The absorbance of the radical intermediates formed in the cell was carried to a Multi-channel Spectrometer (USP-500; UNISOKU Co., Ltd.) through optical fibers. Dosimetry was performed with 5.0×10^{-3} mol dm^{-3} KCNS solutions taking molar absorption coefficient of $(CNS)_2^{-\bullet}$ at 480 nm, $\varepsilon[(CNS)_2^{-\bullet}]_{480} = 7600$ dm^3 mol^{-1} cm^{-1} and $G[(CNS)_2^{-\bullet}] = 2.9 \times 10^{-7}$ mol J^{-1}.

The oxidizing or reducing radicals formed in the reactions of *L*-Lys were titrated by TNM or TMPD, respectively. Pulse radiolysis of the amino acids in the presence of TNM or TMPD forms radical intermediates of the amino acids, which further reduce TNM or oxidize TMPD, and therefore, the yields of intermediate radical species can be quantitated by evaluating the amount of nitroform anion (NF^-) or $TMPD^{+\bullet}$ using $\varepsilon(NF^-)_{350} = 15000$ dm^3 mol^{-1} cm^{-1} or $\varepsilon(TMPD^{+\bullet})_{565} = 12500$ dm^3 mol^{-1} cm^{-1}.

3. RESULTS AND DISCUSSION

3.1 Reaction of *L*-Lys with $^{\bullet}$OH

In the pulse radiolysis of N_2O-saturated aqueous solution of *L*-Lys at pH 7.0, in which $^{\bullet}$OH is a primary reactive radical, transient absorption spectra of intermediate species were observed as shown in Figure 1. A characteristic absorption at 260 nm developed in the first 10 μs and decayed following a second-order kinetics (Figure 1, inset) with a relatively long lifetime, since it was observed over 100 μs after the pulse. Similar transient absorption spectra were also observed in the pH range between 3 and 8.

Pulse radiolysis of N_2O-saturated solution of *L*-Lys in the presence of TMPD at pH 7.0 was also investigated, however formation of $TMPD^{+\bullet}$ was not detected, suggesting oxidizing Lys radicals were not involved in the $^{\bullet}$OH reaction with Lys under neutral conditions. On the other hand, redox titration with TMN as an oxidizing agent showed the formation of NF^- indicating reducing Lys radicals are involved in the $^{\bullet}$OH reaction with Lys at pH 7.0. Buildup of absorption at 350 nm assigned to NF^- was a biphasic kinetics (Figure 2a), and the initial rapid increase in absorption at 350 nm (< 20 μs) was followed by the second slower increase which reaches a plateau in 1 ms after the pulse. Kinetic analysis of the two components for the $^{\bullet}$OH reaction with Lys under various concentrations of TNM revealed that the observed rate constant for formation of the fast growing component (k_{fast}) increased with increasing the concentration of TNM, and approached to a constant value at high TNM concentrations (Figure 2b). Similar kinetic behavior have been reported previously in the redox titration of α-(alkylthio)alkyl radicals by TNM, where a TNM-α-(alkylthio)alkyl radical adduct forms as an intermediate.[26] Therefore, it is presumable that this type of an intermediate is also generated in the reaction of Lys radical ($Lys^{\bullet}$) with TNM, and the formation of NF^- from the Lys-TNM adduct radical becomes a rate-determining step at high concentrations of TNM.

$$Lys^{\bullet} + TNM \rightarrow (Lys\text{-}TNM)^{\bullet} \rightarrow Lys^{+} + NO_2 + NF^-$$

Pseudo-first order kinetic fitting of the fast buildup component in the low concentration range of TNM gave a rate constant of $k_{fast} = 3.9 \times 10^8$ dm^3 mol^{-1} s^{-1} and a G-value for its formation $G_{fast} = 1.7\times 10^{-7}$ mol

J^{-1}. On the other hand, rate constants for formation of the slow component (k_{slow}) were independent of the concentration of TNM over the same concentration range. *G*-value for the slow radical (G_{slow}) and the one-electron transfer rate constant (k_{slow}) were estimated to be $G_{slow} = 0.2 \times 10^{-7}$ mol J^{-1} and $k_{slow} = 1.4 \times 10^{7}$ dm^3 mol^{-1} s^{-1}, respectively.

3.2 Reaction of *L*-Lys with $SO_4^{-\bullet}$

Pulse irradiation of *L*-Lys in Ar-saturated buffer solution containing $K_2S_2O_8$ and *t*-BuOH gave transient species having absorption maxima at around 340 nm and 450 nm as shown in Figure 3. Transient absorption at around 450 nm decayed in a few μs was assigned to $SO_4^{-\bullet}$.[27] The absorption spectra with λ_{max} at 340 nm were observed in the pH range between 2.2 and 9.5, which might be assigned to (1) α-*N*-centered radical (cation) of Lys, (2) ε-*N*-centered radical (cation), or (3) *C*-centered radicals. As a separate experiment, we have carried out pulse radiolysis of *L*-norleucine, which is structurally similar to *L*-Lys but not possessing ε-amino group, in the presence of $K_2S_2O_8$ and *t*-BuOH at pH 7.0, however, any characteristic transient absorption at around 340 nm was not detected, implying that the observed transient absorption at 340 nm can be assigned to a radical cation generated by one-electron oxidation of the ε-amino group of Lys or its deprotonated radical. Much stronger oxidizing ability of $SO_4^{-\bullet}$ [E^0 = ~2.4 V vs NHE] than $^{\bullet}$OH [E^0 = 1.9 V vs NHE] [28] might result in the formation of the *N*-centered radical species, while H-abstraction from the carbon skeleton is a major process in the $^{\bullet}$OH reaction as mentioned above.

The transient species decayed following a first-order kinetics, and the rate constants were dependent on the pH of the solution as shown in Figure 4a. It has been reported that a radical cation formed in the reaction of phenylalanine with $SO_4^{-\bullet}$ undergoes intramolecular electron transfer from the deprotonated carboxyl group to the aminium radical.[29] Therefore, the decay process of the *N*-centered Lys radical may also include such intramolecular electron transfer. Considering that p*K*a for the carboxyl group of *L*-Lys is 2.16, decrease of the rate constant in the pH range below 3 might be due to stabilization of the transient radical species by protonation at the carboxyl group as in the case of phenylalanine. However,

the observed decay rate constants were relatively slow for the proposed intramolecular electron transfer, therefore, it is more likely that the ε-aminium radical is stabilized in the acidic pH region (pH <2), although deprotonated aminyl radical undergoes further oxidation to afford an aldehyde in aqueous solution.

The rate constant for formation of radical intermediate in the reaction of Lys with $SO_4^{-\bullet}$ at pH 7.0 was also evaluated. The characteristic absorption of $SO_4^{-\bullet}$ (λ_{max} = 450 nm) decayed in a few μs after the pulse irradiation and the absorption of the Lys radical then increased, however, the intensity did not reach a plateau level even under the conditions that large amount of Lys was dissolved in the solution. This observation implies that reaction of $^{\bullet}$OH with Lys competes with that with *t*-BuOH, which is added as a scavenger of $^{\bullet}$OH. Such competitive reactions and their rate constants can be analyzed by plotting the optical densities at 340 nm as a function of the concentrations of *t*-BuOH.

$$1 / OD = 1 / OD_0 \{1 + k(t\text{-BuOH} + SO_4^{-\bullet}) [t\text{-BuOH}] / k(\text{Lys} + SO_4^{-\bullet}) [\text{Lys}]\}$$

where, $k(t\text{-BuOH} + SO_4^{-\bullet})$ denotes the rate constant for the reaction of *t*-BuOH with $SO_4^{-\bullet}$ (= 8.0×10^5 dm^3 mol^{-1} s^{-1}),[30] and OD_0 stands for the OD value at 340 nm in the absence of *t*-BuOH. From the plot shown in Figure 4b, the rate constant for the formation of Lys radical intermediate was determined to be $k(\text{Lys} + SO_4^{-\bullet}) = 1.6 \times 10^7$ dm^3 mol^{-1} s^{-1}. The molar extinction coefficient of the Lys radical was also evaluated, assuming the formation and decay of Lys radical is a steady-state sequential reaction. The concentration of Lys radical at the reaction time *t* ($[Lys^{\bullet}]_t$) can be written as

$$[Lys^{\bullet}]_t = [SO_4^{-\bullet}]_0 \{k_f' / (k_f' - k_d)\} \{\exp(-k_d t) - \exp(-k_f' t)\}$$

where k_f' is the rate constant for the pseudo-first-order reaction between $SO_4^{-\bullet}$ and Lys ($k_f' \approx k(\text{Lys} + SO_4^{-\bullet}) [\text{Lys}]_0 = 8.5 \times 10^4$ s^{-1}), and k_d is the rate constant for the unimolecular decay of the Lys radical

($k_d = 4.0 \times 10^4$ s^{-1}). The reaction time when the concentration of Lys radical reaches to its maximum (t_{max}) was thus estimated from the equation above to be $t_{max} = 1.8 \times 10^{-5}$ s. The concentration of $SO_4^{-\bullet}$ generated right after the pulse ($[SO_4^{-\bullet}]_0$) can be calculated from G-value of $SO_4^{-\bullet}$ [$G(SO_4^{-\bullet}) = 3.2 \times 10^{-7}$ mol J^{-1}] and the exposure dose (= 7 Gy). The molecular extinction coefficient of Lys radical at 340 nm (ε_{340}) can be determined with the maximum concentration of Lys radical $[Lys^{\bullet}]_{t\ max} = 1.2 \times 10^{-6}$ mol dm^{-3} and OD_0 obtained from Figure 4b to be $\varepsilon_{340} = 9400$ dm^3 mol^{-1} cm^{-1}.

3.3 Structures of Lys radicals

Structures of the Lys radical intermediates observed in the reaction of Lys and $^{\bullet}OH$ are listed in Figure 5. As have been identified by spin-trapping ESR method, hydrogen abstraction from β, γ and δ-positions of the side chain generates redox-chemically neutral radical intermediates [5] with a total G-value of 4.1×10^{-7} mol J^{-1}. Hydrogen abstraction from α- or ε-CH_2 leading to the corresponding C-centered radicals is also likely, but that from α- or ε-amino group is less possible, because oxidizing Lys radical was not detected during the current pulse radiolysis in the presence of reducing TMPD. It has been reported that the acidity of $-NH_3^+$ group drastically increases by the formation of a radical on the adjoining carbon,[31] thus it can be expected that the amino groups of the α- and ε-C-centered radicals are not protonated under neutral pH conditions.

As has been demonstrated, α-aminyl radical of glycine undergoes further fragmentation into reducing aminomethyl radical ($^{\bullet}CH_2NH_2$) and carbon dioxide radical anion ($CO_2^{-\bullet}$), both of which react with an electron acceptor, 4-carboxybenzophenone with different rate constants.[12] Similarly, fragmentation of the neutral β-C-centered Lys radical would generate reducing $CO_2^{-\bullet}$ ($E^0 = -1.9$ V), and thus the second and slow buildup of NF^- formation that is independent of the TNM concentrations can be explained by the slow formation of $CO_2^{-\bullet}$. Both of the α- and ε-C-centered radicals are expected to be generated as reducing radicals, which are likely to reduce TNM *via* Lys-TNM radical intermediates.

The transient absorption observed in the reaction of Lys with $SO_4^{-\bullet}$ can be assigned to the *ε-N* centered radical or its protonated radical cation generated via one-electron oxidation of *ε*-amino group with a rate constant of k (Lys + $SO_4^{-\bullet}$) = 1.6×10^7 dm^3 mol^{-1} s^{-1}. Its formation is supported by the recent observation that oxidation of Lys by $SO_4^{-\bullet}$ forms covalent adducts between the *ε*-amino group of Lys and the C8 position of guanine to generate spirocyclic products.[22] Considering the apparent first-order decay kinetics of the intermediate radical, the direct oxidation of the *ε*-amino group might be followed by inter-/intra-molecular hydrogen shift to form *C*-centered radicals, as demonstrated earlier in the radical reactions of glycine and other amino acids,[8,9,32] since increased absorption in the short-wavelength range was observed during the pulse radiolysis measurement (Figure 3). Alternatively, further oxidation of *ε*-amino group into aldehyde might be expected, since similar oxidation has been also found in the photocatalytic reaction of *L*-Lys by TiO_2 leading to generation of *L*-pipecolinic acid,[33,34] and in the metal-catalyzed oxidation of Lys to form 2-amino-adipic-semialdehyde as a major product.[35]

4. CONCLUSIONS

In summary, oxidation of a basic amino acid of *L*-Lys by $^{\bullet}OH$ and $SO_4^{-\bullet}$ has been investigated by employing the method of pulse radiolysis. It was demonstrated that hydrogen abstraction by $^{\bullet}OH$ from β, γ, and δ-CH_2 of *L*-Lys generating the redox-neutral *C*-centered radicals is a major process as suggested by previous EPR studies, and that more than two kinds of reducing species are concomitantly generated during the radiolysis. On the other hand, strongly oxidizing $SO_4^{-\bullet}$ induced direct oxidation of Lys, most likely, into the ε-aminyl/aminium radical. Since nuclear DNA is closely associated with Lys- and Arg-rich histone proteins, this type of Lys radical is considered to be a key intermediate radical causing DNA-protein crosslinks, thus the current study will provide useful kinetic parameters for understanding the reaction mechanisms of DNA-protein damages induced under oxidative stresses.

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Figure Captions

Fig. 1. Transient absorption spectra of the intermediates in N_2O-saturated solution of *L*-Lys (5.0×10^{-3} mol dm^{-3}) at pH 7, obtained (●) 1, (■) 10, and (▲) 100 μs after the pulse. The inset shows a reciprocal plot of the absorption at 290 nm.

Fig. 2. (a) Pulse radiolysis of N_2O-saturated aqueous solution of *L*-Lys (5.0×10^{-3} mol dm^{-3}) containing TNM (1.0×10^{-4} mol dm^{-3}) at pH 7. Formation of nitroform anion measured by the absorption at 350 nm as a function of time. (b) Dependence of the observed first-order rate constants for (●) the fast and (○) the slow buildup of nitroform anion as a function of the concentration of TNM.

Fig. 3. Absorption spectra of the intermediates in Ar-saturated solution of *L*-Lys (5.0×10^{-3} mol dm^{-3}) containing $K_2S_2O_8$ (1.0×10^{-2} mol dm^{-3}) and *t*-BuOH (0.10 mol dm^{-3}) at pH 7, obtained (●) 1, (■) 10, and (▲) 50 μs after the pulse. Inset shows decay profiles of the absorption at (■) 340 nm and (●) 450 nm.

Fig. 4. (a) pH-Dependence of the decay rate constants of Lys radical generated in the reaction of *L*-Lys and $SO_4^{-\bullet}$. (b) Plot of 1/OD measured at 340 nm in the pulse radiolysis of *L*-Lys and $SO_4^{-\bullet}$ containing various concentrations of *t*-BuOH.

Fig. 5. Structures of Lys radicals.