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A Pulse Radiolysis Study of the Dynamics of Ascorbic Acid Free Radicals within a Liposomal Environment

Kazuo Kobayashi,*[a] Yumiko Seike,[b] Akinori Saeki,[c] Takahiro Kozawa,[a] Fusako Takeuchi,[d] and Motonari Tsubaki[e]

The dynamics of free-radical species in a model cellular system are examined by measuring the formation and decay of ascorbate radicals within a liposome with pulse radiolysis techniques. Upon pulse radiolysis of an N_2O-saturated aqueous solution containing ascorbate-loaded liposome vesicles, ascorbate radicals are formed by the reaction of $OH^•$ radicals with ascorbate in unilamellar vesicles exclusively, irrespective of the presence of vesicle lipids. The radicals are found to decay rapidly compared with the decay kinetics in an aqueous solution. The distinct radical reaction kinetics in the vesicles and in bulk solution are characterized, and the kinetic data are analyzed.

1. Introduction

Studies of cell-based chemical reactions have primarily focused on the aqueous solution regions of the cell. However, increasing attention has turned toward the chemistry that occurs within lipophilic membranes.[1] For example, the reactions of reactive oxygen species in membranes have been shown to occur readily within cellular membranes and are of significant biological importance.[2] It is important to understand that in vivo antioxidant capacity is determined by the reactivity of antioxidants toward radicals, in addition to several other factors, which include the concentration, tissue distribution, cellular localization, and fate of antioxidant-derived radicals, as well as the interactions with other antioxidants and active metabolic pathways.[3]

Ascorbate (AsH^-) is involved in a wide range of biochemical processes.[4] It serves as a water-soluble antioxidant and is found throughout the human body. Brain and neuroendocrine tissues have the highest AsH^- levels of any organ system. AsH^- plays a unique role as an antioxidant in the brain extracellular microenvironment.[5] AsH^- can potentially protect both the cytosolic and membrane components of cells from oxidative damage. Most oxidized free radicals generated by biological systems can promote the one-electron oxidation of AsH^- to form the ascorbate radical ($As^{•-}$). $As^{•-}$ production and scavenging have been observed to be both associated with, and independent of, enzymatic reactions,[6] and the detection of $As^{•-}$ formation by using EPR spectra provides a measure of oxidative stress.[7] Pulse radiolysis is a powerful tool for investigating the dynamics of $As^{•-}$ in biological systems.[8] In the present work, this technique was extended to the cellular assembly system, and $As^{•-}$ were generated in a model system containing membranes.

[a] Dr. K. Kobayashi, Prof. T. Kozawa
The Institute of Scientific and Industrial Research, Osaka University
Mihogaoka 8-1, Ibaraki, Osaka (Japan)
E-mail: kobayasi@sanken.osaka-u.ac.jp

[b] Y. Seike
Department of Life Science, Faculty of Science
Himeji Institute of Technology, (presently) University of Hyogo
Kamigori-cho, Akou-gun, Hyogo 678-1297 (Japan)

[c] Dr. A. Saeki
Graduate School of Engineering, Osaka University
2-1 Yamadaoka, Suita, Osaka (Japan)

[d] Dr. F. Takeuchi
Institute for Promotion of Higher Education
1-2-1 Tsurukabuto, Nada-ku, Kobe, Hyogo 657-8501 (Japan)

[e] Prof. M. Tsubaki
Kobe University, Graduate School of Science, Department of Chemistry
1-1 Rokkodai-cho, Nada-ku, Kobe, Hyogo 657-8501 (Japan)

2. Results and Discussion

Both oxidative and reductive species were expected to form during the radiolysis of an aqueous solution containing liposome vesicles, in both the exterior and interior aqueous phases of the liposome. Only one type of radical, $As^{•-}$, was generated within the liposomal bilayer under the experimental conditions examined here. Pulse radiolysis of a deaerated aqueous solution produces hydrated electrons, $e^-_{(aq)}$, and $OH^•$ [Eq. (1)]:

$$H_2O \sim\!\!\!\!\rightarrow e^-_{(aq)} + OH^• \quad (1)$$

In the presence of N_2O and 10 mM AsH^-, $As^{•-}$ radical anions are produced in the following reactions [Eqs. (2) and (3)]:

$$e^-_{(aq)} + N_2O + H_2O \rightarrow OH^• + OH^- + N_2 \quad (2)$$

$$OH^• + AsH^- \rightarrow As^{•-} + H_2O \quad (3)$$

In the presence of Cl^-, Cl_2^- (the product of the reaction between Cl^- and $OH^•$) reacts with AsH^- to form $As^{•-}$.[8b] The initial transient increase in absorbance at 360 nm ($A_{360\,nm}$) indicated the formation of $As^{•-}$. The subsequent decay of $As^{•-}$ was ob-

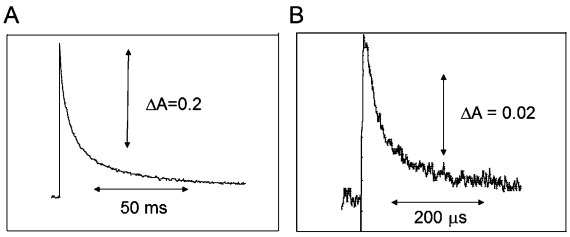

Figure 1. Absorbance changes at 360 nm after pulse radiolysis (160 Gy dose) of aqueous solutions containing A) AsH^- (10 mM) and potassium phosphate buffer (10 mM, pH 7.0) and B) AsH^--loaded liposomes containing phosphate buffer (10 mM, pH 7.0) and NaCl (0.15 M), and saturated with N_2O.

served over the milliseconds timescale (Figure 1 A) and fits well to second-order kinetics.

The pulse radiolysis of an N_2O-saturated aqueous solution containing AsH^--loaded liposomes revealed a similar, but not identical (see below), absorption change (Figure 1 B). The primary species ($e^-_{(aq)}$ and $OH^\bullet$) were formed in both the exterior and interior aqueous phases of the liposome assembly. The $e^-_{(aq)}$ were effectively scavenged by N_2O in this system, as confirmed by the absence of an absorbance change ($\Delta A_{600\,nm}$), owing to the formation of $e^-_{(aq)}$ on the nanoseconds timescale (data not shown). The possibility of a reaction between $e^-_{(aq)}$ and the lipids was excluded, because the intrinsic reactivity of the vesicular amphiphiles with $e^-_{(aq)}$ was generally negligible ($k < 10^7\,M^{-1}s^{-1}$).[9,10] By contrast, the $OH^\bullet$ radicals formed in the interior of the liposomes were scavenged by AsH^- to form $As^{\bullet-}$ [Eq. (3)], because AsH^- (100 mM) was present only within the liposomal bilayer. The initial transient increase in the $A_{360\,nm}$ value indicated the formation of $As^{\bullet-}$, as confirmed by the kinetic differences observed in the obtained spectrum (Figure 2). The spectrum having an absorption maximum at 360 nm was essentially identical to the previously reported spectra of $As^{\bullet-}$ in aqueous solution.[8a] These findings indicated that $As^{\bullet-}$ radical anions in the unilamellar vesicles were generated exclusively by the method used here.

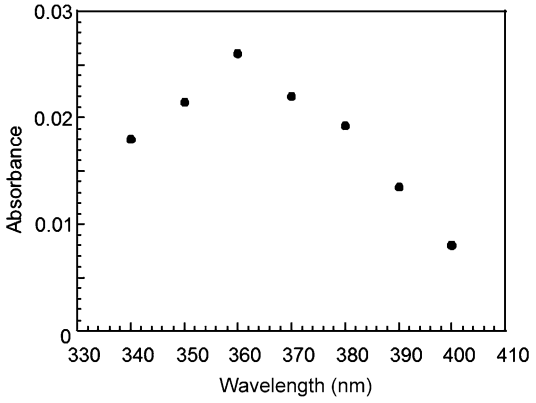

Figure 2. Kinetic difference spectrum 50 ns after pulse radiolysis of AsH^--loaded liposomes. The experimental conditions were the same as described for Figure 1.

These results raised an important question: why did the $As^{\bullet-}$ radical anions form so efficiently within the liposomes? The absorbance changes of $As^{\bullet-}$ in the liposomes corresponded to approximately 10% of the pulse radiolysis of the homogenous aqueous solution of AsH^-, although the liposomes constituted less than 2% of the total volume. Under the experimental conditions used, the $OH^\bullet$ radicals generated in the bulk solution could not reach the AsH^- species trapped within the liposomes. Most of the $OH^\bullet$ species generated in the interior of the liposomes were able to react with lecithin of the liposome at a high rate ($k \approx 5\times10^8\,M^{-1}s^{-1}$).[10] The $OH^\bullet$ most likely attacked the head group of lecithin at the surface of the bilayer, but not the hydrocarbon chain of the bilayer.[10] The resulting radical species further reacted with AsH^- efficiently. The role of AsH^- as a radical scavenger in the system was confirmed by examining the effects of AsH^--free liposomes on the pulse radiolysis experiments. Under these conditions, AsH^- anions are present in the exterior of liposome. The pulse radiolysis of an N_2O-saturated aqueous solution containing 0.1 M AsH^- and AsH^--free liposomes induced the $OH^\bullet$ species to react with both AsH^- in the exterior of the liposome and the liposome (AsH^--free). The absorbance changes at 360 nm observed during the formation and decay of $As^{\bullet-}$, however, were unaffected by the further addition of AsH^--free liposomes (Figure 3). These results strongly

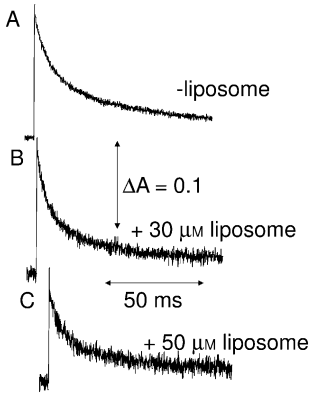

Figure 3. Absorbance changes after pulse radiolysis of AsH^--free liposomes containing AsH^- (0.1 M) and with N_2O-saturated potassium phosphate buffer (10 mM, pH 7.0).

suggested that the radicals formed by the reaction with lecithin, a secondary reaction of $OH^\bullet$, were scavenged by AsH^-. This process occurred rapidly and efficiently, even though AsH^- ions were localized at the polar surface of the membrane.[2c]

Surprisingly, the kinetic behavior of $As^{\bullet-}$ in the liposome system was quite distinct from the behavior observed in bulk solutions (Figures 1 and 2). The $As^{\bullet-}$ radical anions in the liposomes decayed rapidly over the microseconds timescale (Figure 1 B). The decay of $As^{\bullet-}$ was insensitive to the solution pH, unlike the corresponding decay observed in aqueous solutions (Figure 4 B). In the absence of other reactants, the $As^{\bullet-}$ species disproportionated to form AsH^- and dehydroascorbate (A) through a proton-dependent second-order process that in-

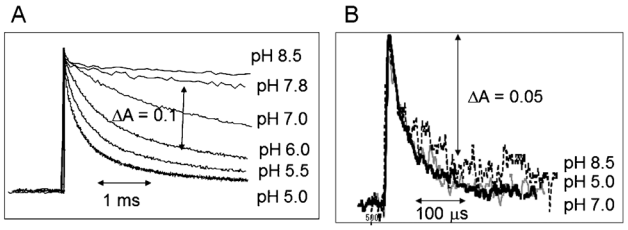

Figure 4. pH-dependent absorbance changes at 360 nm after pulse radiolysis of an aqueous solution containing A) AsH^- (10 mM) and potassium phosphate buffer (10 mM), and B) AsH^--loaded liposomes containing N_2O-saturated phosphate buffer (10 mM).

volved the transient dimer $(As_2)^{2-}$ [Eqs. (4)–(6)]:[8b]

$$2\,As^{\bullet-} \rightleftharpoons (As_2)^{2-} \quad (4)$$

$$(As_2)^{2-} + H^+ \rightarrow AsH^- + A \quad (5)$$

$$(As_2)^{2-} + H_2O \rightarrow AsH^- + A + OH^- \quad (6)$$

The decay of $As^{\bullet-}$ increased rapidly as the solution acidity increased from pH 8 to 5 (Figure 4 A). Ascorbyl radicals are surprisingly long-lived in anhydrous acetonitrile. Labile protons are required for radical disproportionation.[12] Therefore, the pH-independent process of $As^{\bullet-}$ decay in the liposome system (Figure 4 B) suggested that the rate-limiting disproportionation of $As^{\bullet-}$ involved the formation of the transient dimer [Eq. (4)], which then reacted rapidly with H^+ [Eq. (5)]. Alternatively, labile protons might be present in the liposome system.

The differences between the decay rates might be explained in terms of the restricted diffusion of the radicals within the liposome. Experimental evidence for this effect was obtained from intravesicular kinetic processes in which one reactant was confined to the interior of the vesicular assembly. Neither $As^{\bullet-}$ nor $AsH^{\bullet-}$ species could pass through the vesicle membranes.[10] Assuming that $As^{\bullet-}$ decayed through a disproportionation mechanism, the decay can be expressed by Equation (7):

$$\frac{\partial c(r,t)}{\partial t} = D\nabla^2 c(r,t) - k[c(r,t)]^2 \quad (7)$$

where $c(r, t)$ is the concentration of $As^{\bullet-}$ species inside the liposome at time t and at a distance r from the center of liposome, and D is the diffusion coefficient. At time $t = 0$, no delocalization of the confined species was assumed. The gray and black lines in Figure 5 represent the experimental and simulated curves, respectively. In the simulation, the parameters of the diffusion coefficient of the $As^{\bullet-}$ radicals were $D = 5.0 \times 10^{-5}$ $cm^2 s^{-1}$, $r = 100$ nm, $c_0 = 1.5 \times 10^{-4}$ M, $k = 2 \times 10^4$ $M^{-1} s^{-1}$ (c_0 is the initial concentration). Among these parameters, the liposome size, diffusion coefficient, and the initial concentrations were not important for the decay process. This result was consistent with the experimental observation that the liposome size was 50–1000 nm and that the radical concentration did not appreciably affect the decay kinetics. In contrast, the rate constant was an important factor for the decay process. Notably, the simulated rate constant (2×10^4 $M^{-1} s^{-1}$) is one order of magnitude smaller than the apparent rate constant observed for an aqueous solution at pH 7.0 (3×10^6 $M^{-1} s^{-1}$). The simulated rate constant for the reaction described by Equation (4) in a homogeneous aqueous solution was 7.8×10^7 $M^{-1} s^{-1}$[8b], which is much higher than that obtained.

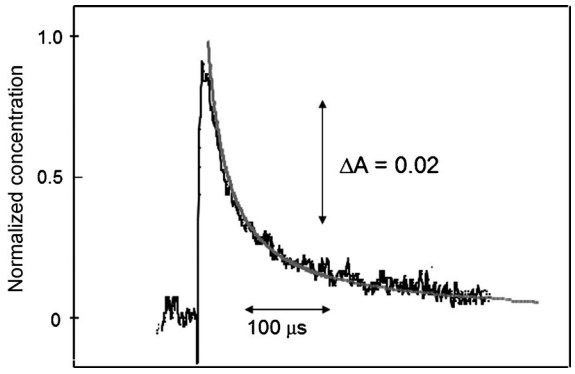

Figure 5. Comparison of the experimental (black) and simulated (gray) decay kinetics of $As^{\bullet}$ species within liposomes.

3. Conclusions

We examined the intravesicular kinetic reactions of $As^{\bullet-}$ radicals. The radicals in vesicles decayed rapidly compared with the radicals in aqueous solution. Pulse radiolytic studies of these systems were used to simulate the radiation-induced biochemical reactions in media with a similar composition to that of cells. The radiolytic pulses initiated novel reactions in the nanospaced regions in the presence of organized molecular assemblies.

Experimental Section

Preparation of Ascorbate-Loaded Liposomes

AsH^--loaded vesicles were prepared by using the freeze–thaw method.[12] A mixture of dipalmitoyl-L-α-phosphatidyl-DL-glycerol, dipalmitoyl-L-α-phosphatidyl choline, and cholesterol (0.2:1:1 molar ratio; Presome PPG1, Nippon Fine Chemical Co., Osaka, Japan) was suspended in potassium phosphate buffer (20 mM, pH 7.0, 10 mL) containing NaCl (0.15 M) and AsH^- (0.1 M). A freeze–thaw protocol was subsequently undertaken by placing the sample in liquid N_2 until frozen and then thawing several times. The liposomes thus obtained were dialyzed overnight against phosphate buffer (10 mM, pH 7.0) containing NaCl (0.15 M) and AsH^- (0.1 M). The multilamellar vesicles were extruded repeatedly (31 times) through a polycarbonate filter (Nucleopore, Pleasanton, CA) mounted in the mini-extruder (LipsoFast Basic, Avestin Inc., Ottawa, Canada). The pore size of the filters used were 50, 100, 200, 400, 800, and 1000 nm. The liposome samples obtained were gel-filtered through an Ampure SA-column (GE Healthcare Life Sciences) equilibrated with phosphate buffer (10 mM, pH 7.0) containing NaCl (0.15 M) at room temperature. The sizes of unilamellar liposomes were measured using a JEOL JEM-122EX electron microscope. Leakage of AsH^- out of the liposome was not observed within 24 h at room temperature; this was determined by checking

the reduction of cytochrome *c*. Inclusion of AsH^- in the vesicle was confirmed by full reduction of cytochrome *c* upon addition of 1% Triton X-100.

Pulse Radiolysis

The liposome samples used for the pulse radiolysis studies were prepared as follows: N_2O gas was bubbled through solutions containing phosphate buffer (10 mM, pH 7.0) and NaCl (0.15 M) for 5 min. An aliquot (20 μL) of the liposome solution was added to the solution. The system contained dipalmitoyl-L-α-phosphatidyl-DL-glycerol, dipalmitoyl-L-α-phosphatidyl choline, and cholesterol (3:1:1, total concentration ≈1 mM). Pulse radiolysis was performed using an electron linear accelerator at the Institute of Scientific and Industrial Research, Osaka University.[8d–f] The pulse width and energy were 8 ns and 27 MeV, respectively. The dose was in the range of 15–400 Gy. A 1 kW xenon lamp was used as a light source. After passing through quartz cells with an optical path length of 1 cm, the transmitted light was analyzed and its intensity monitored by a fast spectrophotometric system composed of a Nikon monochromator, an R-928 photomultiplier and a Unisoku data analysis system.

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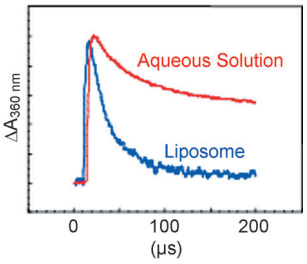

K. Kobayashi, Y. Seike, A. Saeki, T. Kozawa, F. Takeuchi, M. Tsubaki*

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