•OH and $O_2^{\bullet-}$ Generation in Aqueous C_{60} and C_{70} Solutions by Photoirradiation: An EPR Study

Yoko Yamakoshi, Shoko Sueyoshi, Kiyoshi Fukuhara, and Naoki Miyata*

Division of Organic Chemistry
National Institute of Health Sciences
1-18-1 Kamiyoga, Setagaya, Tokyo 158-8501, Japan

Toshiki Masumizu and Masahiro Kohno

Analytical Instruments Division, JEOL Ltd.
3-1-2 Musashino, Akishima, Tokyo 196-0021, Japan

Received July 8, 1998

Fullerenes (C_{60}, C_{70}, etc.), carbon allotropes with a globular conjugated π-electron system, possess unique physicochemical properties. The biological activity of fullerenes has attracted considerable attention with regard to the development of novel pharmaceuticals.[1] C_{60} and its derivatives have been reported to promote chondrogenesis,[2] and to exhibit enzyme-inhibiting activity[3] and radical-quenching activity.[4] These activities may be useful in some pharmaceutical applications. Fullerenes are also very strong photosensitizing agents[5] and may exhibit bioactivity against biomolecules with light, which could lead to their wider application as pharmaceuticals. Along these lines, the photoinduced DNA-cleaving and lipid peroxidation activities have been studied.[6] In these reports, the oxidative damage of biomolecules was thought to be primarily caused by singlet oxygen ($^1O_2$) produced by photoexcited fullerenes since photosensitization by fullerenes is frequently attributed to the generation of $^1O_2$, that is, the ground state of C_{60} ($^1C_{60}$) is excited by visible light irradiation to give a singlet excited state ($^1C_{60}$*) followed by conversion to a triplet state ($^3C_{60}$*) through an intersystem crossing in high quantum yield (nearly 100%). Subsequently, $^3C_{60}$* transfers energy to $^3O_2$ to generate $^1O_2$ (*type II* energy-transfer pathway).[5a,c] Essentially the same process has been noted for C_{70}.[5b] Photoexcited fullerenes also react with various electron donors.[7] $^3C_{60}$* has a higher electron-accepting ability than $^1C_{60}$, and electron-donating compounds such as amines[7b,h,k,m], antioxidants,[7d,g] etc. can reduce $^3C_{60}$* to give the C_{60} radical anion ($C_{60}^{\bullet-}$) via a *type I* electron-transfer pathway. $C_{60}^{\bullet-}$ generation with light in the presence of an electron donor was demonstrated by UV−vis and EPR methods,[8] and the involvement of $C_{60}^{\bullet-}$, rather than $^1O_2$, in the DNA cleavage was recently proposed.[6e] In this paper, we report photoinduced DNA cleavage by C_{60} and C_{70} in the presence of NADH, a common reductant *in vivo*, and the detection of reduced active oxygen species ($O_2^{\bullet-}$ and •OH) under photoirradiation by the EPR spectroscopic method coupled with a spin-trapping agent. These oxyl radicals may be generated by electron transfer from $C_{60}^{\bullet-}$ to molecular oxygen, and may be the ultimate active species for DNA cleavage.

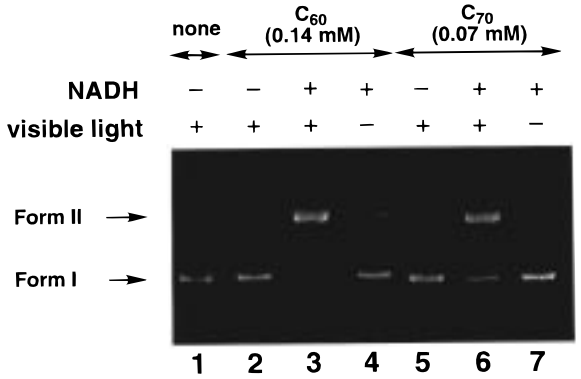


Figure 1. Photoinduced DNA cleavage by C_{60} and C_{70}. The pBR322 supercoiled plasmid (Wako) was incubated with each chemical in TDC buffer for 4 h at 37 °C under irradiation with a 300-W reflector lamp. Lanes 1−3 and 5−6, incubation under visible light irradiation: lane 1, pBR322 DNA with 1.25% PVP; lane 2, with 0.14 mM C_{60}; lane 3, with 0.14 mM C_{60} and 10 mM NADH; lane 5, with 0.07 mM C_{70}; lane 6, with 0.07 mM C_{70} and 10 mM NADH. Lanes 4 and 7, incubation in the dark: lane 4, pBR322 DNA with 0.14 mM C_{60} and 10 mM NADH; lane 7 with 0.07 mM C_{70} and 10 mM NADH.

The effect of a reductant on the DNA-cleaving activity of C_{60} or C_{70} was first examined using pBR322 supercoiled DNA. C_{60} (>99.98%, Terms) and C_{70} (>99%, MER) were dissolved in water with detergent, poly(vinylpyrrolidone) (PVP).[9] NADH served as the reductant and a 300-W reflector lamp served as the source of visible light irradiation. In the presence of NADH (10 mM), pBR322 was cleaved into form II (nicked DNA) under photoirradiation (Figure 1, lane 3 for C_{60} and lane 6 for C_{70}). The effect of NADH was dose-dependent, and no DNA cleavage occurred in the absence of NADH (Figure 1, lanes 2 and 5). Photoirradiation was required for DNA cleavage by either of these compounds (Figure 1, lanes 4 and 7). NADH should reduce $^3C_{60}$* ($^3C_{70}$*) to $C_{60}^{\bullet-}$ ($C_{70}^{\bullet-}$), which may be essential for $O_2^{\bullet-}$ generation.[10] To prove that $O_2^{\bullet-}$ is involved in DNA cleavage,

* To whom correspondence should be addressed. Phone: +81-3-3700-1141 (ext. 222). Fax: +81-3-3700-9418. E-mail: miyata@nihs.go.jp.

(1) Jensen, A. W.; Wilson, S. R.; Schuster, D. I. *Bioorganic Med. Chem.* **1996**, *4*, 767.

(2) (a) Tsuchiya, T.; Yamakoshi, Y. N.; Miyata, N. *Biochem. Biophys. Res. Commun.* **1995**, *206*, 885. (b) Tsuchiya, T.; Oguri, I.; Yamakoshi, Y. N.; Miyata, N. *Fullerene Sci. Technol.* **1996**, *4*, 989.

(3) HIV-1 protease inhibition: (a) Friedman, S. H.; DeCamp, D. L.; Sijbesma, R. P.; Srdanov, G.; Wudl, F.; Kenyon, G. L. *J. Am. Chem. Soc.* **1993**, *115*, 6506. (b) Toniolo, C.; Bianco, A.; Maggini, M.; Scorrano, G.; Prato, M.; Marastoni, M.; Tomatis, R.; Spisani, S.; Palú, R.; Blair, E. D. *J. Med. Chem.* **1994**, *37*, 4558. Glutathione *S*-transferase inhibition: (c) Iwata, N.; Mukai, T.; Yamakoshi, Y.; Hara, S.; Yanase, T.; Shoji, M.; Endo, T.; Miyata, N. *Fullerene Sci. Technol.* **1998**, *6*, 213.

(4) (a) Chiang, L. Y.; Lu, F.-J.; Lin, J.-T. *J. Chem. Soc., Chem. Commun.* **1995**, 1283. (b) Lai, Y.-L.; Chiou, W.-Y.; Chiang, L. Y. *Fullerene Sci. Technol.* **1997**, *5*, 1057.

(5) (a) Arbogast, J. W.; Darmanyan, A. P.; Foote, C. S.; Rubin, Y.; Diedrich F. N.; Alvarez, M. M.; Anz, S. J., Whetten, R. L. *J. Phys. Chem.* **1991**, *95*, 11. (b) Arbogast, J. W.; Foote, C. S. *J. Am. Chem. Soc.* **1991**, *113*, 8886. (c) Nagano, T.; Arakane, K.; Ryu, A.; Masunaga, T.; Shinmoto, K.; Mashioko, S.; Hirobe, M. *Chem. Pharm. Bull.* **1994**, *42*, 2291.

(6) (a) Tokuyama, H.; Yamago, S.; Nakamura, E.; Shiraki, T.; Sugiura, Y. *J. Am. Chem. Soc.* **1993**, *115*, 7918. (b) Boutorine, A. S.; Tokuyama, H.; Takasugi, M.; Isobe, H.; Nakamura, E.; Hélène, C. *Angew. Chem., Int. Ed. Engl.* **1994**, *33*, 2462. (c) Irie, K.; Nakamura, Y.; Ohigashi, H.; Tokuyama, H.; Yamago, S.; Nakamura, E. *Biosci. Biotechnol. Biochem.* **1996**, *60*, 1359. (d) Nakamura, E.; Tokuyama, H.; Yamago, S.; Shiraki, T.; Sugiura, Y. *Bull. Chem. Soc. Jpn.* **1996**, *69*, 2143. (e) An, Y.-Z.; Chen, C.-H. B.; Anderson, J. L.; Sigman, D. S.; Foote C. S.; Rubin, Y. *Tetrahedron* **1996**. *52*, 5179**.** (f) Yamakoshi, Y.; Yagami, T.; Sueyoshi, S.; Miyata, N. *J. Org. Chem.* **1996**, *61*, 7236. (g) Sera, N,; Tokiwa, H.; Miyata, N. *Carcinogenesis* **1996**, *17*, 2163.

(7) (a) Krusic, P. J.; Wasserman, E.; Parkinson, B. A.; Malone, B.; Holler, E. R., Jr.; Keizer, P. N.; Morton, J. R.; Preston, K. F. *J. Am. Chem. Soc.* **1991**, *113*, 6274. (b) Arbogast, J. W.; Foote, C. S.; Kao, M. *J. Am. Chem. Soc.* **1992**, *114*, 2277. (c) Ruebsam, M.; Dinse, K.-P.; Plueschau, M.; Fink, J.; Krätschmer, W.; Fostiropoulos, K.; Taliani, C. *J. Am. Chem. Soc.* **1992**, *114*, 10059. (d) Hwang, K. C.; Mauzerall, D. *J. Am. Chem. Soc.* **1992**, *114*, 9705. (e) Hwang, K. C.; Mauzerall, D. *Nature* **1993**, *361*, 138. (f) Osaki, T.; Tai, Y.; Tazawa, M.; Tanemura, S.; Inukai, K.; Ishiguro, K.; Sawaki, Y.; Saito, Y.; Shinohara, H.; Nagashima, H. *Chem. Lett.* **1993**, 789. (k) Guldi, D. M.; Asmus, K.-D. *J. Phys. Chem.* **1997**, *101*, 1472. (l) Mikami, K.; Matsumoto, S.; Ishida, A.; Takamura, S.; Suenobu, T.; Fukuzumi, S. *J. Am. Chem. Soc.* **1995**, *117*, 11134. (m) Fukuzumi, S.; Suenobu, T.; Kawamura, S.; Ishida, A.; Mikami, K. *Chem. Commun.* **1997**, 291.

(8) (a) Brezová, V.; Staško, A.; Rapta, P.; Domschke, G.; Bartl, A.; Dunsch, L. *J. Phys. Chem.* **1995**, *99*, 16234. (b) Brezová, V.; Guegel, A.; Rapta, P.; Staško, A. *J. Phys. Chem.* **1996**, *100*, 13252. (c) Staško, A.; Brezová, V.; Rapta, P.; Biskupic, S.; Guegel, A. *Res. Chem. Intermed.* **1997**, *23*, 453.

(9) Yamakoshi, Y.; Yagami, T.; Fukuhara, K.; Sueyoshi, S.; Miyata, N. *J. Chem. Soc., Chem. Commun.* **1994**, 517.

10.1021/ja9823969 CCC: $15.00
Published on Web 11/13/1998

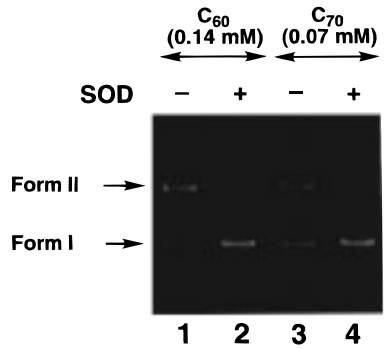
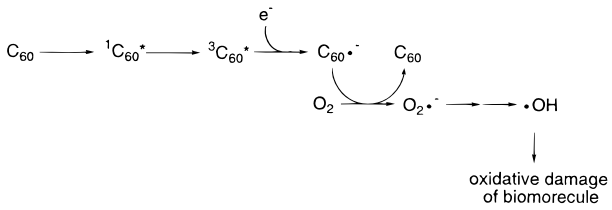


Figure 2. Effects of SOD on photoinduced DNA cleavage by C_{60} and C_{70}. The procedure for photoirradiation was the same as that in Figure 1. The duration of photoirradiation was 2 h. Lanes 1−4, incubation under visible light irradiation: lane 1, pBR322 DNA with 0.14 mM C_{60} and 10 mM NADH; lane 2, with 0.14 mM C_{60}, 10 mM NADH and 0.04 units/mL of SOD; lane 3, with 0.07 mM C_{70} and 10 mM NADH; lane 4, with 0.07 mM C_{70}, 10 mM NADH and 0.04 units/mL of SOD.

Scheme 1. Possible Pathway for the Generation of Oxyl Radicals by Photoexcited C_{60}



experiments were performed using superoxide dismutase (SOD). With the addition of 0.01 μg/mL (0.04 units/μL) SOD, the formation of nicked DNA decreased significantly (Figure 2, lane 2 for C_{60} and lane 4 for C_{70}). $^1O_2$ scavengers, such as NaN_3, 2,5-dimethylfuran, and L-histidine, and D_2O, which prolongs the lifetime of $^1O_2$, did not have any effects. Thus, the photoinduced damage of DNA would appear to occur via a *type I* electron-transfer pathway in which superoxide ($O_2^{\bullet-}$) is generated as an intermediate.

To confirm the generation of $O_2^{\bullet-}$ and •OH, EPR was carried out using PVP-solubilized C_{60} and C_{70} under irradiation with a 300-W reflector lamp. 5,5-Dimethyl-1-pyrroline-*N*-oxide (DMPO) served as the spin-trapping agent to detect •OH and $O_2^{\bullet-}$. •OH was detected by adding NADH (10 mM) and then DMPO (0.75 M) to an aqueous solution of C_{60}, followed by photoirradiation for 60 s. Four characteristic peaks were detected for the DMPO adduct of •OH (DMPO−OH) (Figure 3c). The peak height was dependent on the duration of irradiation and on the amount of C_{60} and NADH. No peaks (or only small peaks) appeared without photoirradiation or in the absence of NADH (Figures 3a and b, respectively). To detect the generation of $O_2^{\bullet-}$, DMSO (5.9 M) was added to the previous mixture to scavenge •OH. At 5 s of photoirradiation, characteristic peaks for the DMPO adduct of $O_2^{\bullet-}$ (DMPO−OOH) could be seen instead of those for the DMPO−OH (Figure 3d). These peaks completely disappeared with the addition of SOD. Peak height was dose-dependently increased by C_{60} and NADH. In the absence of NADH, no peaks were detected for DMPO−OOH. Similar results were obtained for C_{70} (Figure 3e).

The present findings indicate that $O_2^{\bullet-}$ and •OH are efficiently formed by photoexcited fullerenes in aqueous solution (Scheme 1).[11] Electrons are transferred from NADH to photoexcited fullerenes and then to O_2 to generate $O_2^{\bullet-}$. These oxyl radicals were observed even at a low NADH concentration (0.08 mM), and thus the reductive activation of O_2 by photoexcited fullerenes should be possible under physiological conditions. $O_2^{\bullet-}$ and subsequently produced •OH may be the primary factors for the biological damage caused by fullerenes under photoirradiation.[12]

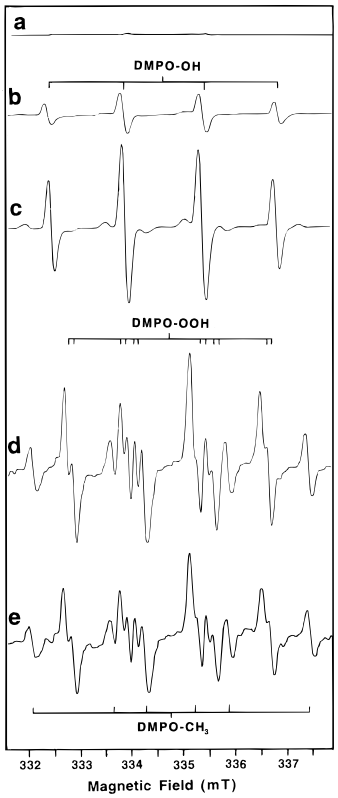


Figure 3. EPR spectra of DMPO adducts of active oxygen species (•OH and $O_2^{\bullet-}$) generated in aqueous solutions of C_{60} and C_{70} under irradiation with a 300-W reflector lamp: (a) with C_{60} 0.12 mM, PVP 0.4%, DMPO 0.75 M, NADH 10 mM (under dark conditions); (b) with C_{60} 0.12 mM, PVP 0.4%, DMPO 0.75 M (under visible light irradiation for 60 s). (c) with C_{60} 0.12 mM, PVP 0.4%, DMPO 0.75 M, NADH 10 mM (under visible light irradiation for 60 s); (d) with C_{60} 0.12 mM, PVP 0.4%, DMSO 5.9M, DMPO 0.75 M, NADH 10 mM (under visible light irradiation for 5 s); (e) with C_{70} 0.12 mM, PVP 0.4%, DMSO 5.9M, DMPO 0.75 M, NADH 10 mM (visible light irradiation for 5 s). The hyperfine splittings were $a_N = a_H^\beta = 1.48$ mT for DMPO−OH, $a_N = 1.37$, $a_H^\beta = 1.09$, $a_H^\alpha = 0.10$ mT for DMPO−OOH and $a_N = 1.57$ mT and $a_H^\beta = 2.23$ mT for DMPO−CH_3. These coupling constants are in good agreement with the values reported in ref 13.

Acknowledgment. This paper is dedicated to the memory of Professor Toru Koizumi, deceased January 8, 1998. We are grateful to Professors Hideo Utsumi (Kyushu University) and Andrej Staško (Slovak Technical University) for fruitful discussions and valuable comments. This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture, (Nos. 05233103, 08772053 (Y.Y.), 08878074 (N.M.), and 09772037 (Y.Y.)) and by a Grant-in-Aid for Scientific Research from the Ministry of Health and Welfare (Y.Y.).

Supporting Information Available: Experimental and detailed EPR spectroscopic data (13 pages, print/PDF). See any current masthead page for ordering information and Web access instructions.

JA9823969

(10) Photoinduced electron transfer from NADH analogues to photoexcited C_{60} has recently been reported: Fukuzumi, S.; Suebobu, T.; Patz, M.; Hirasaka, T.; Itoh, S.; Fujitsuka, M.; Ito, O. *J. Am. Chem. Soc.* **1998**, *120*, 8060.

(11) •OH generation, see: Halliwell, B.; Gutteridge, J. M. C. In *Methods in Enzymology*, Packer, L., Glazer A. N., Eds.; Academic Press: San Diego, 1990; Vol. 186, Part B, p 1−85.

(12) Although we cannot yet exclude the possibility that $^1O_2$ is involved in the oxidative DNA damage caused by photoexcited fullerenes, the generation of $^1O_2$ was not observed under our experimental conditions by EPR spectroscopy using TEMPO (2,2,6,6-tetramethyl-4-piperidone) as an $^1O_2$-trapping agent.

(13) (a) Buettner, G. R.; Mason, R. P. In *Methods in Enzymology*; Packer, L., Glazer A. N., Eds.; Academic Press: San Diego, 1990; Vol. 186, Part B, p 127−133. (b) Kohno, M., Mizuta, Y.; Masumizu, T.; Makino, K. *Bull. Chem. Soc. Jpn.* **1994**, *67*, 1085.