DNA Cleavage via Superoxide Anion Formed in Photoinduced Electron Transfer from NADH to γ-Cyclodextrin-Bicapped C_{60} in an Oxygen-Saturated Aqueous Solution

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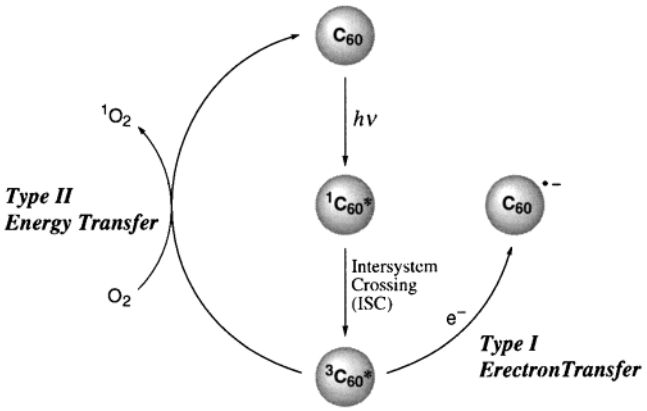
γ-Cyclodextrin-bicapped C_{60} (C_{60}/γ-CyD) shows an efficient DNA cleaving-activity in the presence of NADH (β-nicotinamide adenine dinucleotide, reduced form) in an O_2-saturated aqueous solution under visible-light irradiation. No DNA cleavage has been observed without NADH under experimental conditions that are otherwise the same, although singlet oxygen ($^1O_2$) has been detected by the ESR spin-trapping of the C_{60}/γ-CyD-O_2 system. This indicates that neither triplet excited state of C_{60}/γ-CyD ($^3C_{60}$*/γ-CyD) nor $^1O_2$ produced via an energy transfer from $^3C_{60}$*/γ-CyD to O_2 is an actual reactive species, which is responsible for the DNA damage under the present experimental conditions. In the presence of NADH, photoinduced electron transfer from NADH to $^3C_{60}$*/γ-CyD occurs to yield two equivalents of the radical anion ($C_{60}^{\bullet-}$/γ-CyD), which exhibits its characteristic NIR band at 1080 nm. The dynamics of the photoinduced electron transfer have been examined by monitoring the decay of triplet−triplet absorption band at 740 nm and concomitant rise of the NIR absorption band at 1080 nm due to $C_{60}^{\bullet-}$/γ-CyD with use of the laser flash photolysis for the C_{60}/γ-CyD-NADH system. In the presence of O_2, $C_{60}^{\bullet-}$/γ-CyD disappears via the electron transfer to O_2 and an electron transfer from NADH to $^1O_2$ to produced $O_2^{\bullet-}$. The formation of $O_2^{\bullet-}$ has been confirmed by the spin trap with DEPMPO (5-diethoxyphosphoryl-5-methyl-1-pyrroline-*N*-oxide), which is an efficient $O_2^{\bullet-}$-trapping agent. The reorganization energy for the reduction of O_2 to $O_2^{\bullet-}$ is evaluated as 43.4 kcal mol^{-1}, which agrees with the literature value determined directly for the self-exchange between $^{36}O_2^{\bullet-}$ and $^{32}O_2$. This indicates that the electron transfer from $C_{60}^{\bullet-}$/γ-CyD to O_2 proceeds via an outer-sphere pathway. The $O_2^{\bullet-}$ thus produced gives H_2O_2, ultimately yielding hydroxyl radical, which is shown to be an actual DNA-cleaving reagent.

Introduction

Fullerenes, such as C_{60} and C_{70}, are known to have strong photosensitizing activities[1,2] and the potential utility of fullerenes as pharmaceuticals has been extensively explored in recent years.[3,4] Fullerenes, which are sensitive to light at wavelengths longer than 500 nm, are expected to be an effective photodynamic therapy agent because bodily tissues are most transparent in this region of wavelengths.[5] C_{60} and its derivatives are reported to promote chondrogenesis,[6] exhibiting enzyme-inhibiting activity[7] and radical-quenching activity.[8] In particular, the DNA-cleaving and lipid peroxidation activities of fullerenes have attracted considerable attention.[9−11] At first, it was presumed that the mechanism of DNA cleavage involves photoexcitation of the fullerene group followed by sensitized formation of singlet oxygen ($^1O_2$) which would then cleave DNA.[8,9] Photoirradiation of C_{60} results in the formation of the singlet excited state $^1C_{60}$*, which undergoes efficient intersystem crossing (ISC) to give the triplet excited state $^3C_{60}$* (Scheme 1).[12] The $^3C_{60}$* thus formed efficiently transfers energy to molecular oxygen to give $^1O_2$ (type II energy-transfer pathway).

SCHEME 1



The possible intermediacy of $^1O_2$ was examined in some detail by comparing the reactivity of the fullerene-oligonucleotide

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linked system with a similarly linked eosin-origonucleotide.[10] A singlet oxygen quencher, sodium azide, was found to inhibit the eosin-oligonucleotide cleavage, but not the fullerene-oligonucleotide cleavage.[10] This suggests that the fullerene-oligonucleotide cleavage does not involve the singlet oxygen mechanism, but rather some other mechanism must be involved.[10] The most probable mechanism may be an electron transfer because the reduction potential of $^3C_{60}$* is significantly high (+1.14 V vs SCE in benzonitrile),[13] and an electron transfer from reductants, such as amines and antioxidants, to $^3C_{60}$* occurs to give the radical anion of C_{60} ($C_{60}^{\bullet-}$) (type I electron-transfer pathway). Formation of $C_{60}^{\bullet-}$ in the photoinduced electron-transfer reactions from a variety of electron donors to $^3C_{60}$* has been demonstrated with use of the transient vis-NIR spectroscopy as well as the ESR technique.[14−19] Foote et al. have reported that the $^3C_{60}$* directly oxidizes guanine in a DNA stack via the type I electron-transfer mechanism because the oxidation potential of a guanosine derivative is located at 1.26 V which is close to the reduction potential of $^3C_{60}$* (1.14 V).[20,21] When photoinduced electron transfer occurs from electron donors to $^1C_{60}$*, the back electron transfer to the triplet excited-state rather than to the ground state can occur provided that the triplet excited state is lower in energy than the radical ion pair state.[22] In such a case, formation of $^1O_2$ may occur via photoinduced electron transfer and subsequent energy transfer from $^3C_{60}$* to oxygen.[17] Thus, there are several possible pathways for the DNA-cleaving process involving $^3C_{60}$*: via superoxide anion, singlet oxygen, or direct oxidation of DNA. However, the poor solubility of fullerenes to water has so far precluded the detailed mechanistic studies of C_{60}-photosensitized DNA damage. The preparation of a water-soluble C_{60} complex has been achieved by using γ-cyclodextrin (γ-CyD) as a host in the complex where the fullerene core is embedded between the cavities of two γ-CyD molecules.[23] Although the reactivity of the fullerene in this complex is noticeably lower than that in the free molecule, C_{60}/γ-CyD is still susceptible for the sensitizing and redox processes.[24,25]

We have previously reported photocleavage of DNA in the presence of NADH (β-nicotinamide adenine dinucleotide, reduced form) in water, when C_{60} or C_{70} used as a sensitizer was dissolved with use of poly(vinylpyrrolidone) (PVP).[26] However, the actual intermediate for the photocleavage of DNA has yet to be clarified.[11a]

We report herein that photoinduced DNA cleavage occurs efficiently by C_{60}/γ-CyD in an O_2-saturated aqueous solution containing NADH which is the most important redox coenzyme acting as the source of electrons in the living system. Detailed spectroscopic and kinetic studies using a laser flash photolysis technique, detection of superoxide ($O_2^{\bullet-}$) with use of DEPMPO,[27] and the effects of hydroxyl radical scavengers on DNA cleavage are now performed to provide confirmative mechanistic insight into the C_{60}/γ-CyD-photosensitized DNA damage by oxygen in the presence of NADH.

Experimental Section

Materials. C_{60} (>99.99% pure) was purchased from MER Co., Tucson, AZ. γ-Cyclodextrin was obtained commercially from Wako Pure Chemical Ind. Ltd., Japan and dried under vacuum prior to use. NADH (β-nicotinamide adenine dinucleotide, reduced form) was purchased from Sigma Chemical Co. DNA pBR322 (0.51 μg μL^{-1}) was purchased from Wako Pure Chemical Ind. Ltd., Japan. 2,2,6,6-Tetramethyl-4-piperidone (TEMP) used as a trapping agent of singlet oxygen was purchased from Aldrich. 5-Diethoxyphosphoryl-5-methyl-1-pyrroline-*N*-oxide (DEPMPO) used as an $O_2^{\bullet-}$ -trapping agent was obtained commercially from OXIS International, Inc. and used as received.

Preparation of γ-Cyclodextrin-Bicapped C_{60} (C_{60}/γ-CyD). γ-Cyclodextrin-bicapped C_{60} (C_{60}/γ-CyD) was prepared by ball milling of the mixture containing 100 mg (0.14 mmol) of C_{60} and 900 mg (0.70 mmol) of γ-Cyclodextrin (γ-CyD) for 2 h at room temperature, followed by an addition of 10 mL of water.[28,29] Aqueous solutions containing C_{60}/γ-CyD were filtered with a membrane filter (pore size: 0.22 μm). The concentration of C_{60}/γ-CyD was estimated by using the ϵ value of 51 900 M^{-1} cm^{-1} at 330 nm determined for the cyclohexane solution.[28a,29]

DNA Cleavage. The 30 μL of aqueous solution of DNA pBR322 (0.51 μg μL^{-1}) was diluted by adding 270 μL of water. Typically, 10 μL of aqueous solution of C_{60}/γ-CyD (1.8×10^{-4} M), 10 μL of NADH (4.0×10^{-2} M), 12 μL of aqueous solution of DNA pBR322 (2.84×10^6 D; 0.051 μg μL^{-1}), and 8 μL of phosphate buffer (250 mM, pH 7.4) were mixed in a micro test tube under dark conditions. Samples were incubated under irradiation with a 300-W reflector lamp for 1 h at 273 K, mixed with 10 μL of loading buffer (0.1% bromophenol blue and 30% glycerol in TBE buffer), and loaded onto a 1% agarose gel containing ethidium bromide (1 μg mL^{-1}). The gels were run at a constant voltage of 70 V for 2 h in TBE buffer, washed with distilled water, visualized under a UV transilluminator, and photographed using an instant camera.

ESR Measurements. In a typical experiment of the ESR measurements for the detection of $^1O_2$, 50 μL of aqueous solution of C_{60}/γ-CyD (2.3×10^{-4} M), 50 μL of 250 mM phosphate buffer solution (pH 7.4), 100 μL of water, and 50 μL of aqueous solution of TEMP (0.5 M) were mixed in a LABOTEC LLC-04B ESR sample tube. The ESR samples were then irradiated with a 300-W reflector lamp prior to the measurements. ESR spectra were measured with a JEOL JES−RE1XE and were recorded under nonsaturating microwave power conditions. The magnitude of the modulation was chosen to optimize the resolution and the signal-to-noise ratio (S/N) of the observed spectra. The g values were calibrated using an Mn^{2+} marker. The detection of $O_2^{\bullet-}$ with use of DEPMPO as a $O_2^{\bullet-}$-trapping agent was carried out in a similar manner.

Spectroscopic Measurements. Typically, to a deaerated 50 mM phosphate buffer solution (pH 7.4; 3 mL) of C_{60}/γ-CyD (1.3×10^{-4} M) in a quartz cuvette (10 mm i.d.) under an atmospheric pressure of argon was added NADH (1.5×10^{-4} M), and the solution was irradiated with a Xe lamp (Ushio Model V1-501C) through a UV cut-off filter (Toshiba Y-47) transmitting $\lambda > 470$ nm at 298 K for 2 h. The vis-NIR spectra were measured on a Shimadzu UV-3100PC spectrophotometer.

Laser Flash Photolysis. The nanosecond time-resolved absorption spectra were measured using second-harmonic generation (532 nm) of a Nd:YAG laser (QuantaRay GCR-130, fwhm 6 ns) as an excitation source. For the transient absorption spectra in the NIR region (600−1600 nm), a Ge avalanche photodiode (APD) (Hamamatsu Photonics, B2834) was employed as a detector for monitoring light from a pulsed Xe flash lamp.[30] The long time scale phenomena up to millisecond-region were measured using an InGaAs−PIN photodiode (Hamamatsu Photonics, G5125−10) or a Si-APD (Hamamatsu Photonics, S5343) as a detector for monitoring light from a continuous Xe-lamp (150 W).[31] Because the purple solution of C_{60}/γ-CyD in water disappeared by each laser shot (532 nm; 7 mJ) in the presence of NADH, the transient spectra were recorded using

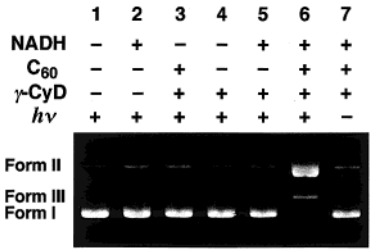


Figure 1. DNA-cleaving activities of C_{60}/γ-CyD in phosphate buffer (50 mM, pH 7.4) after visible-light irradiation with a 300-W reflector lamp for 1 h at 273 K. $[C_{60}] = 4.6 \times 10^{-5}$ M; [γ-CyD] $= 9.2 \times 10^{-5}$ M; [NADH] $= 1.0 \times 10^{-2}$ M; $[O_2] = 1.3 \times 10^{-3}$ M.

fresh solutions in each laser excitation. All experiments were performed at 295 K. The concentration of O_2 in the solution was adjusted by argon, air, or O_2 purging for 15 min prior to the measurements ($[O_2] = 0$, 2.7×10^{-4}, or 1.3×10^{-3} M, respectively). Pseudo-first-order rate constants were determined by a least-squares curve fit using an Apple Macintosh personal computer. The first-order plots of $\ln(A_\infty - A)$ vs time (A_∞ and A are the final absorbance and the absorbance during the reaction, respectively) were linear for three or more half-lives with the correlation coefficient $\rho > 0.999$.

Quantum Yield Determination. A standard actinometer (potassium ferrioxalate)[32] was used for the quantum yield determination of the photochemical reactions of C_{60}/γ-CyD and NADH. A square quartz cuvette (10 mm i.d.) which contained a 50 mM phosphate buffer solution (pH 7.4; 3 mL) of C_{60}/γ-CyD (1.3×10^{-4} M) and NADH ($1.0 \times 10^{-5} - 1.3 \times 10^{-2}$ M) was irradiated with monochromatized light of $\lambda = 532$ nm from a Shimadzu RF-5000 fluorescence spectrophotometer. The solution was first evacuated with ultra sonic irradiation and then argon gas was bubbled for 15 min. Under the conditions of actinometry experiments, both the actinometer and C_{60}/γ-CyD absorbed essentially all the incident light. The light intensity of monochromatized light of $\lambda = 532$ nm was determined as 2.2×10^{-8} einstein s^{-1} with the slit width of 10 nm. The photochemical reaction was monitored by using a Shimadzu UV-3100PC spectrophotometer The quantum yields were determined from the increase in absorbance due to $C_{60}^{\bullet-}/\gamma$-CyD ($\lambda_{max} = 1080$ nm, $\epsilon = 1.2 \times 10^4$ M^{-1} cm^{-1})[33] and the decrease from the absorbance due to NADH ($\lambda = 380$ nm, $\epsilon = 1.2 \times 10^3$ M^{-1} cm^{-1}).

Results and Discussion

Photoinduced DNA Cleavage in the Aqueous C_{60}/γ-CyD-NADH-O_2 System. The DNA-cleaving activity of C_{60}/γ-CyD was examined using pBR322 supercoiled DNA. In the presence of NADH as an electron donor, pBR322 was efficiently cleaved into form II (nicked DNA) and form III (linear DNA) after 1 h visible-light irradiation of a phosphate buffer solution (50 mM, pH 7.4) containing C_{60}/γ-CyD and molecular oxygen with use of a 300-W reflector lamp (Figure 1, lane 6). The effect of NADH as well as C_{60}/γ-CyD was dose-dependent (see Supporting Information). NADH, C_{60}/γ-CyD, or γ-CyD alone shows no DNA-cleaving activity even in the presence of O_2 under irradiation (Figure 1, lane 2, 3, or 4, respectively). NADH in the presence of γ-CyD also shows no DNA-cleaving activity (Figure 1, lane 5). Under dark conditions, no DNA cleavage occurs even in the presence of all the components, i.e., C_{60}/γ-CyD, NADH, and O_2 (Figure 1, lane 7).

As shown in Scheme 1, photoirradiation of the aqueous C_{60}/γ-CyD solution in the absence of an electron donor under aerobic conditions may result in the formation of singlet oxygen ($^1O_2$) via energy transfer from the triplet excited state of C_{60} to molecular oxygen (type II pathway). In fact, $^1O_2$ was detected by the ESR spin-trapping with use of TEMP (2,2,6,6-tetramethyl-4-piperidone), which is a $^1O_2$-trapping agent.[34] TEMP reacts with $^1O_2$ to give a $^1O_2$-adduct, TEMPO.[34] The characteristic three ESR signals of TEMPO with a_N value of 15.8 G was observed under visible-light irradiation of the aqueous C_{60}/γ-CyD-O_2 system as shown in Figure 2a.

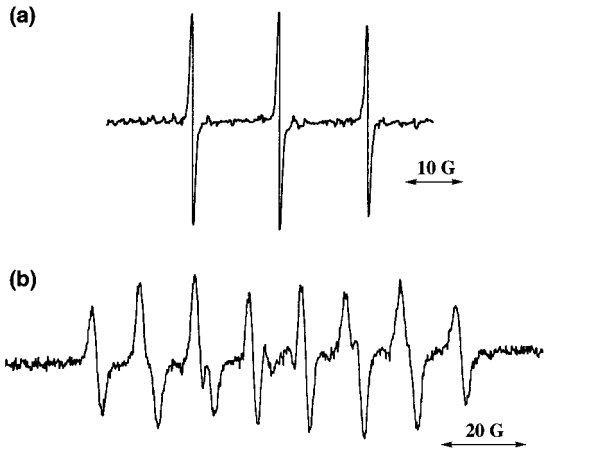


Figure 2. X-band ESR spectra of (a) TEMPO formed after visible-light irradiation of a phosphate buffer solution (50 mM, pH 7.4) of C_{60}/γ-CyD (4.6×10^{-5} M) and TEMP (0.1 M) in the presence of O_2 for 180 s at 298 K and (b) DEPMPO−OOH formed after irradiation of a phosphate buffer solution (50 mM, pH 7.4) of C_{60}/γ-CyD (4.6×10^{-5} M), NADH (1.0×10^{-3} M), and DEPMPO (1.0×10^{-2} M) in the presence of O_2 for 60 s at 298 K.

Although $^1O_2$ was suggested to have a DNA-cleaving activity,[8,9] no photoinduced DNA cleavage occurs in the aqueous C_{60}/γ-CyD-O_2 system under our experimental conditions (Figure 1, lane 3). In the presence of NADH, however, superoxide anion ($O_2^{\bullet-}$) was detected by the ESR spin-trapping with use of DEPMPO (5-diethoxyphosphoryl-5-methyl-1-pyrroline-*N*-oxide) as an $O_2^{\bullet-}$ -trapping agent.[27] DEPMPO reacts with $O_2^{\bullet-}$ to give the $O_2^{\bullet-}$ -adduct, DEPMPO−OOH, which shows a characteristic ESR signal different from that of •OH-adduct, DEPMPO−OH. Figure 2b shows the ESR spectrum of DEPMPO−OOH observed after visible-light irradiation of an O_2-saturated phosphate buffer solution (50 mM, pH 7.4) containing C_{60}/γ-CyD, NADH, and DEPMPO at 298 K. These results indicate that the DNA cleavage occurs via $O_2^{\bullet-}$ formation via type I electron-transfer pathway, where the triplet excited state $^3C_{60}$*/γ-CyD formed by visible-light irradiation undergoes one-electron reduction by NADH to give the radical anion $C_{60}^{\bullet-}/\gamma$-CyD. Because the oxidation potential of $C_{60}^{\bullet-}/\gamma$-CyD (−0.56 V vs SCE)[35] is more negative than the reduction potential of O_2 (−0.40 V vs SCE),[36] electron transfer from $C_{60}^{\bullet-}/\gamma$-CyD to O_2 to give $O_2^{\bullet-}$ is energetically feasible.

Photoinduced One-Electron Reduction of C_{60}/γ-CyD. Visible light irradiation of a deaerated aqueous solution containing C_{60}/γ-CyD and NADH results in the formation of the radical anion of C_{60}/γ-CyD ($C_{60}^{\bullet-}/\gamma$-CyD). No reaction occurs in the dark. The formation of $C_{60}^{\bullet-}/\gamma$-CyD is detected by the typical NIR band at 1080 nm as shown in Figure 3. The $C_{60}^{\bullet-}/\gamma$-CyD generated in the photochemical reaction is stable in deaerated aqueous solution, and from the spectral titration (inset of Figure 3) is established the stoichiometry of the reaction as shown in eq 1, where NADH acts as a two-electron donor to reduce 2 equiv of C_{60}/γ-CyD to $C_{60}^{\bullet-}/\gamma$-CyD.[37]

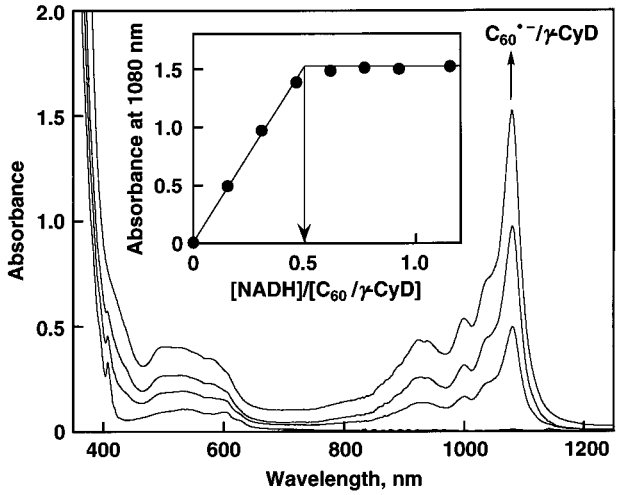


Figure 3. Vis-NIR spectra of deaerated phosphate buffer (50 mM, pH 7.4) of C_{60}/γ-CyD (1.3×10^{-4} M) with NADH (0, 2.0×10^{-5}, 4.0×10^{-5}, 1.5×10^{-4} M) after visible-light irradiation with a Xe lamp ($\lambda > 470$ nm) for 2 h at 298 K. Inset: Plot of the absorbance at 1080 nm vs [NADH]/[C_{60}/γ-CyD].

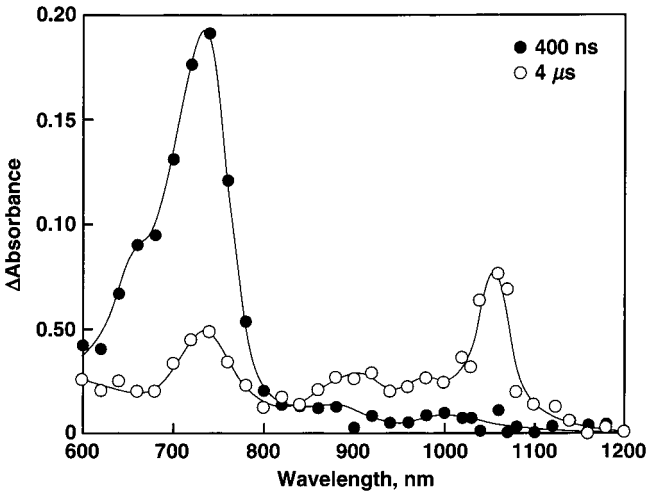
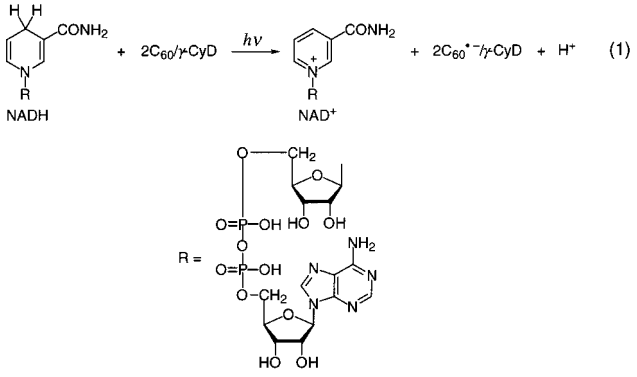


Figure 4. Transient absorption spectra observed in the photoreduction of C_{60}/γ-CyD (5.5×10^{-5} M) by NADH (4.0×10^{-3} M) at 400 ns and 4 μs after laser excitation in deaerated phosphate buffer (50 mM, pH 7.4) at 295 K.



The singlet excited state of C_{60} produced initially upon irradiation is known to be efficiently converted to the triplet excited state by the fast intersystem crossing.[12,13] The transient absorption spectra in the visible and NIR region are observed by the laser flash photolysis of a deaerated aqueous solution of C_{60}/γ-CyD in the presence of NADH with 532 nm laser light as shown in Figure 4.

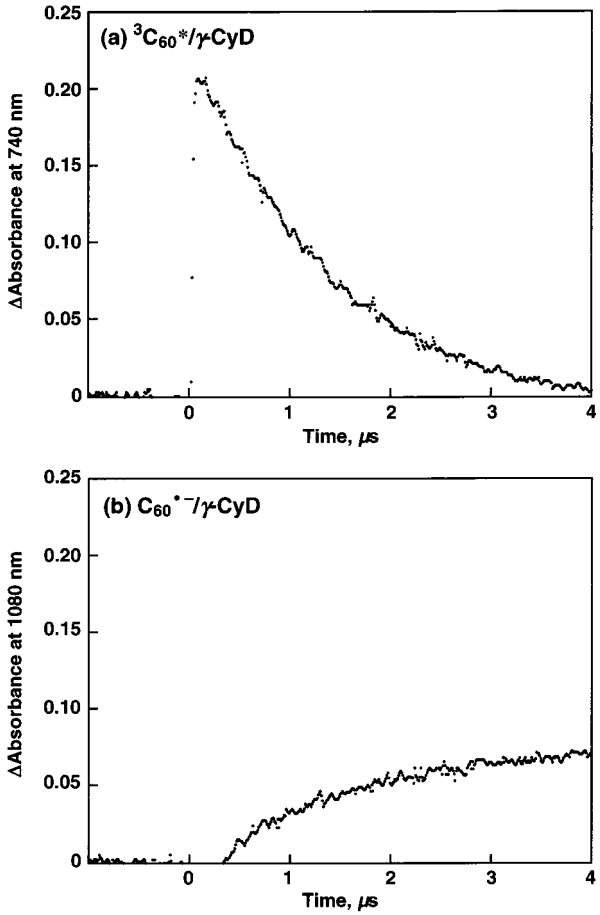
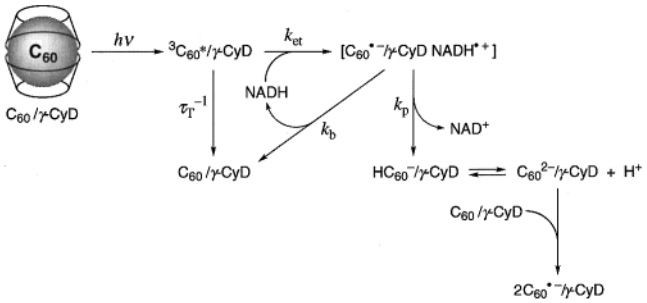


Figure 5. (a) Decay of the absorbance at 740 nm due to $^3C_{60}*/\gamma$-CyD and (b) rise of the absorbance at 1080 nm due to $C_{60}^{\bullet-}/\gamma$-CyD observed in the photoreduction of C_{60}/γ-CyD (5.5×10^{-5} M) by NADH (8.0×10^{-3} M) after laser excitation in deaerated phosphate buffer (50 mM, pH 7.4) at 295 K.

SCHEME 2



The transient absorption band at 740 nm appearing immediately after nanosecond laser pulse excitation is attributed to the triplet−triplet absorption band of $^3C_{60}*/\gamma$-CyD. The decay of the absorption band of $^3C_{60}*/\gamma$-CyD is accompanied by appearance of a new absorption band at 1080 nm which is diagnostic of $C_{60}^{\bullet-}/\gamma$-CyD. The decay of the absorbance at 740 nm due to $^3C_{60}*/\gamma$-CyD obeys pseudo-first-order kinetics, coinciding with the rise of the absorbance at 1080 nm due to $C_{60}^{\bullet-}/\gamma$-CyD as shown in Figure 5.

The mechanism for generation of two equivalents of $C_{60}^{\bullet-}/\gamma$-CyD in eq 1 may be essentially the same as reported for the photoreduction of C_{60} by an NADH model compound,[38] as shown in Scheme 2. First photoinduced electron transfer from NADH to $^3C_{60}*/\gamma$-CyD (k_{et}) occurs to give the radical ion pair [$C_{60}^{\bullet-}/\gamma$-CyD $NADH^{\bullet+}$] in competition with the decay of $^3C_{60}*/$

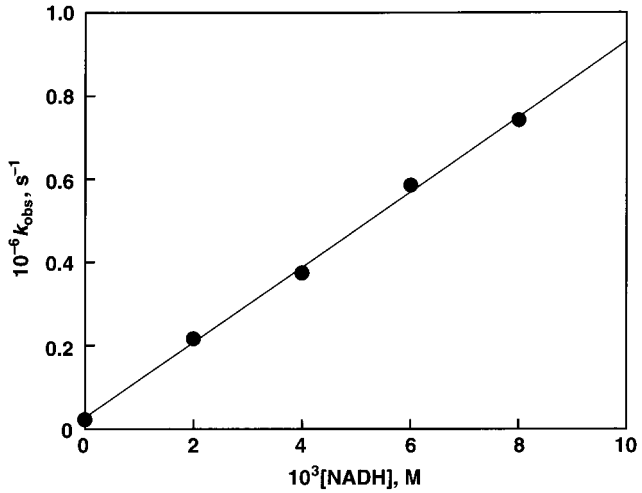


Figure 6. Plots of k_{obs} vs [NADH] in the photoreduction of C_{60}/γ-CyD (5.5×10^{-5} M) by [NADH] in deaerated phosphate buffer (50 mM, pH 7.4) at 298 K.

γ-CyD to the ground state (τ_T^{-1}). This is followed a hydrogen (or proton and electron) transfer from $NADH^{\bullet+}$ to $C_{60}^{\bullet-}$/γ-CyD (k_p) to produce HC_{60}^{-}/γ-CyD and NAD^+ in competition with the back electron transfer to the reactant pair (k_b), The subsequent facile electron transfer from C_{60}^{2-}/γ-CyD being in equilibrium with HC_{60}^{-}/γ-CyD to C_{60}/γ-CyD leads to formation of two equivalents of $C_{60}^{\bullet-}$/γ-CyD (Scheme 2)

According to Scheme 2, the decay of $^3C_{60}$*/γ-CyD and the concomitant appearance of $C_{60}^{\bullet-}$/γ-CyD are derived as given by eqs 2 and 3, respectively

$$[^3C_{60}*/\gamma\text{-CyD}] = [^3C_{60}*/\gamma\text{-CyD}]_0 \exp[-(k_{et}[NADH] + \tau_T^{-1})t] \quad (2)$$

$$[C_{60}^{\bullet-}/\gamma\text{-CyD}] = [C_{60}^{\bullet-}/\gamma\text{-CyD}]_\infty (1 - \exp[-(k_{et}[NADH] + \tau_T^{-1})t]) \quad (3)$$

In eq 3, $[C_{60}^{\bullet-}/\gamma\text{-CyD}]_\infty$ is the concentration of $C_{60}^{\bullet-}$/γ-CyD produced as the final product in eq 1 and this is given by eq 4.

$$[C_{60}^{\bullet-}/\gamma\text{-CyD}]_\infty = \frac{2k_{et}\tau_T[NADH]}{1 + k_{et}\tau_T[NADH]}\left(\frac{k_p}{k_p + k_b}\right)[^3C_{60}*/\gamma\text{-CyD}]_0 \quad (4)$$

According to eq 3, the pseudo-first-order decay rate constant of $^3C_{60}$*/γ-CyD (k_{obs}) increases linearly with an increase in the concentration of NADH with an intercept, which corresponds to τ_T^{-1}. This is confirmed as a linear plot of k_{obs} vs [NADH] in Figure 6. From the slope is obtained the k_{et} value as 1.0×10^8 M^{-1} s^{-1}. The τ_T value is obtained from the intercept as 33 μs which agrees with the literature value.[11,12]

By application of the steady-state approximation to the reactive species in Scheme 2, the dependence of the quantum yield (Φ) of formation of $C_{60}^{\bullet-}$/γ-CyD on [NADH] can be derived as given by eq 5 which predicts an increase in Φ with [NADH] to reach the limiting value Φ_∞ ($= 2k_p/(k_p + k_b)$).[39]

$$\Phi = \frac{2k_{et}\tau_T[NADH]}{1 + k_{et}\tau_T[NADH]}\left(\frac{k_p}{k_p + k_b}\right) \quad (5)$$

This relation is confirmed experimentally as shown in Figure 7a. The dependence of Φ on [NADH] is converted to a linear relation between Φ^{-1} and $[NADH]^{-1}$ (Figure 7b). From the slope and intercept are obtained the Φ_∞ and $k_{et}\tau_T$ values as 1.03 and 3.56×10^3 M^{-1}, respectively. The $k_p/(k_p + k_b)$ value is determined from the Φ_∞ value as 0.515. The k_{et} value is determined from the $k_{et}\tau_T$ value as 1.08×10^8 M^{-1} s^{-1} which agrees with the value (1.0×10^8 M^{-1} s^{-1}) determined directly from the appearance of $C_{60}^{\bullet-}$/γ-CyD in Figure 6. Such an agreement confirms the validity of Scheme 2.

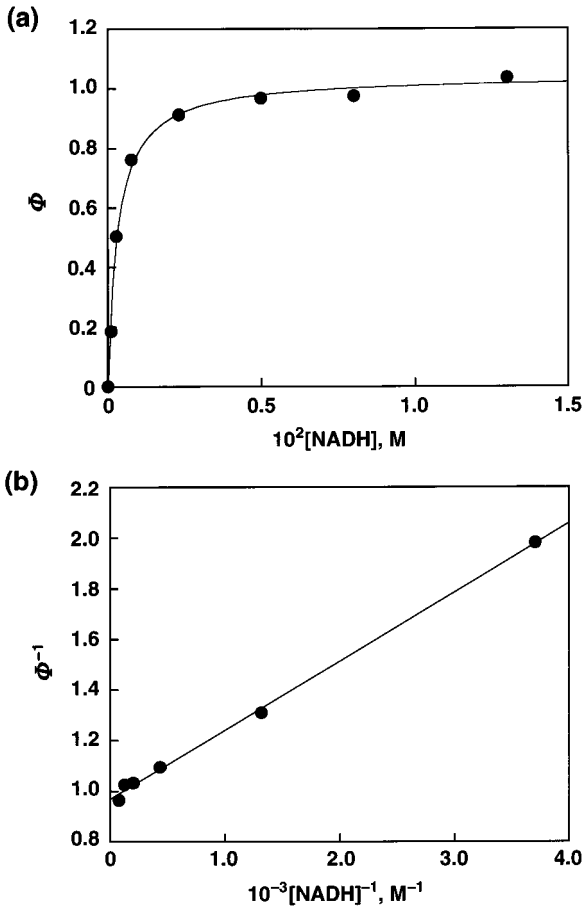


Figure 7. (a) Dependence of the quantum yield (Φ) on [NADH] for the photoreduction of C_{60}/γ-CyD (1.3×10^{-4} M) by NADH in deaerated phosphate buffer (50 mM, pH 7.4) at 298 K. (b) Plot of Φ^{-1} vs $[NADH]^{-1}$.

Electron Transfer from $C_{60}^{\bullet-}$/γ-CyD to O_2. When O_2 is introduced to the aqueous solution containing $C_{60}^{\bullet-}$/γ-CyD formed in the photoreduction of C_{60}/γ-CyD by NADH, the absorption band at 1080 nm due to $C_{60}^{\bullet-}$/γ-CyD has disappeared immediately. Because the oxidation potential of $C_{60}^{\bullet-}$/γ-CyD (-0.56 V vs SCE)[34] is more negative than the reduction potential of O_2 (-0.40 V vs SCE),[35] electron transfer from $C_{60}^{\bullet-}$/γ-CyD to O_2 takes place to give C_{60}/γ-CyD and superoxide anion ($O_2^{\bullet-}$). In fact, $O_2^{\bullet-}$ was detected under visible-light irradiation of the aqueous C_{60}/γ-CyD-NADH-O_2 system by the ESR spin-trapping with use of DEPMPO as an $O_2^{\bullet-}$-trapping agent (vide supra). The long time scale observations of the time profiles of $C_{60}^{\bullet-}$/γ-CyD in the presence of different concentrations of molecular oxygen by the laser flash photolysis are shown in Figure 8 where the decay rate of the absorption due to $C_{60}^{\bullet-}$/γ-CyD at 1080 nm increases with increasing $[O_2]$.

The mechanism for the C_{60}/γ-CyD-catalyzed generation of $O_2^{\bullet-}$ with NADH is shown in Scheme 3, where an energy transfer form $^3C_{60}$*/γ-CyD to O_2 (k_{EN}) and an electron transfer from $C_{60}^{\bullet-}$/γ-CyD to O_2 (k_{et}') are added to the mechanism for

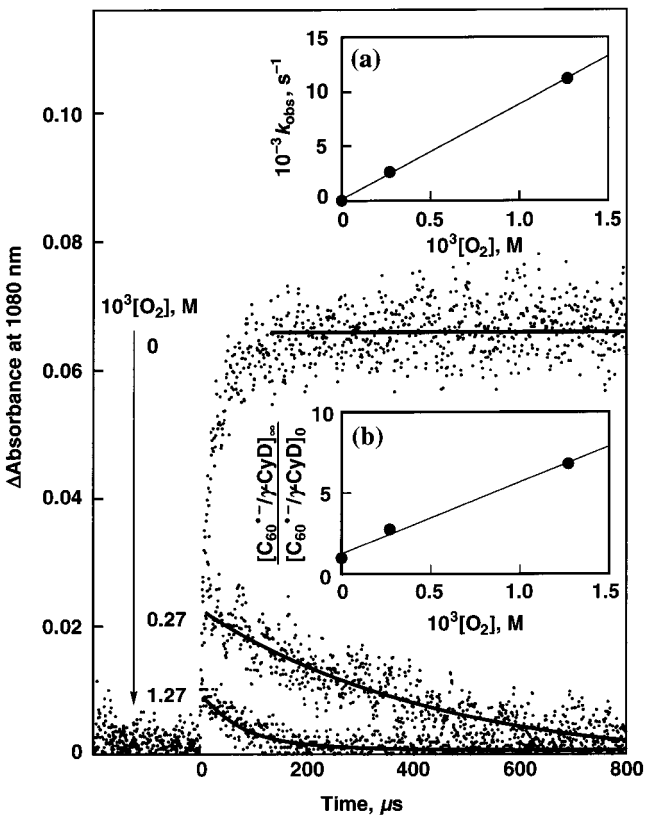
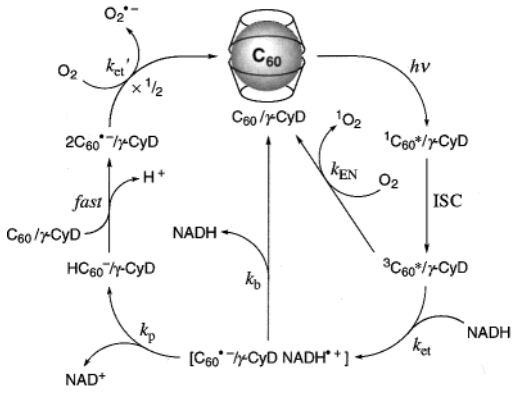


Figure 8. Time course changes of the absorbance at 1080 nm due to $C_{60}^{\bullet-}/\gamma$-CyD (5.5×10^{-5} M) in the presence of different concentration of O_2 in phosphate buffer (50 mM, pH 7.4) at 295 K. $[O_2] = 0$, 2.7×10^{-4}, 1.3×10^{-3} M. Insets: (a) plot of k_{obs} vs $[O_2]$; (b) plot of $[C_{60}^{\bullet-}/\gamma\text{-CyD}]_\infty/[C_{60}^{\bullet-}/\gamma\text{-CyD}]_0$ vs $[O_2]$.

SCHEME 3



the photoreduction of C_{60} by NADH without O_2 (Scheme 2). According to Scheme 3, the decay of $C_{60}^{\bullet-}/\gamma$-CyD in the

$$[C_{60}^{\bullet-}/\gamma\text{-CyD}] = [C_{60}^{\bullet-}/\gamma\text{-CyD}]_0 \exp(-k_{et}'[O_2]t) \quad (6)$$

presence of O_2 is given by eq 6 where $[C_{60}^{\bullet-}/\gamma\text{-CyD}]_0$ is the initial concentration of $C_{60}^{\bullet-}/\gamma$-CyD in the presence of O_2 and is given by eq 7. In accordance with eq 6, the decay of $C_{60}^{\bullet-}/$

$$[C_{60}^{\bullet-}/\gamma\text{-CyD}]_0 = \frac{2k_{et}\tau_T[\text{NADH}][^3C_{60}^*/\gamma\text{-CyD}]_0}{1 + k_{et}\tau_T[\text{NADH}] + k_{EN}\tau_T[O_2]}\left(\frac{k_p}{k_p + k_b}\right) \quad (7)$$

γ-CyD obeys pseudo-first-order kinetics and the pseudo-first-order rate constant (k_{obs}) increases linearly with increasing $[O_2]$

TABLE 1: Observed Quantum Yields (Φ_{obs}) of Consumption of NADH in C_{60}/γ-CyD(1.3×10^{-4} M)-Photosensitized Reduction of O_2 by NADH in a Phosphate Buffer Solution (50 mM, pH 7.4) at 298 K and the Calculated Quantum Yields (Φ_{calc})

[NADH], M	$[O_2]$, M	Φ_{obs}	Φ_{calc}
7.5×10^{-4}	2.7×10^{-4}	0.22	0.16
9.8×10^{-4}	2.7×10^{-4}	0.24	0.19
1.2×10^{-3}	2.7×10^{-4}	0.25	0.22
5.5×10^{-4}	1.3×10^{-3}	0.12	0.10
8.3×10^{-4}	1.3×10^{-3}	0.16	0.13
1.1×10^{-3}	1.3×10^{-3}	0.18	0.17
1.4×10^{-3}	1.3×10^{-3}	0.20	0.20

as shown in the inset of Figure 8. From the slope of the linear plot of k_{obs} vs $[O_2]$ is obtained the k_{et}' value as 8.1×10^6 M^{-1} s^{-1}. The ratio of $[C_{60}^{\bullet-}/\gamma\text{-CyD}]_\infty$ in the absence of O_2 to $[C_{60}^{\bullet-}/\gamma\text{-CyD}]_0$ in the presence of O_2 is obtained from eqs 4 and 7 as given by eq 8. A linear correlation between $[C_{60}^{\bullet-}/\gamma\text{-CyD}]_\infty/[C_{60}^{\bullet-}/\gamma\text{-CyD}]_0$ and $[O_2]$ with the intercept of unity is confirmed as shown in Figure 8b. From the slope of the linear correlation in Figure 8b is obtained the k_{EN} value as 1.3×10^9 M^{-1} s^{-1}. This value agrees with the reported k_{EN} value (1.6×10^9 M^{-1} s^{-1}).[16]

$$[C_{60}^{\bullet-}/\gamma\text{-CyD}]_\infty/[C_{60}^{\bullet-}/\gamma\text{-CyD}]_0 = 1 + k_{EN}\tau_T[O_2]/(1 + k_{et}\tau_T[\text{NADH}]) \quad (8)$$

The reorganization energy for the self-exchange reaction of $O_2^{\bullet-}/O_2$ (λ_{11}) is derived from the Marcus equation,[40] as given by eq 9,[41] where $\Delta G^\ddagger$ is the activation free energy and λ_{22} is the reorganization energy for the self-exchange reaction of $C_{60}^{\bullet-}/C_{60}$. The λ_{22} value has previously been determined as 14.3 kcal mol^{-1}.[42]

$$\lambda_{11} = 2(2\Delta G^\ddagger - \Delta G^0_{et} + 2[\Delta G^\ddagger(\Delta G^\ddagger - \Delta G^0_{et})]^{1/2}) - \lambda_{22} \quad (9)$$

The $\Delta G^\ddagger$ value is obtained from the k_{et}' value using eq 10, where Z is the collision frequency taken as 1×10^{11} M^{-1} s^{-1} and the other notations are conventional. The λ_{11} value is determined as 43.4 kcal mol^{-1} from the $\Delta G^\ddagger$ and ΔG^0_{et} values using eq 9, This value agrees with the reported value of 45.5 kcal mol^{-1}

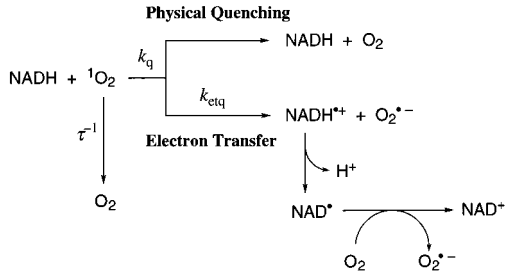
$$\Delta G^\ddagger = 2.3RT \log(Z/k_{et}') \quad (10)$$

which was directly determined utilizing ^{18}O as a probe for the self-exchange reaction between $^{36}O_2^{\bullet-}$ and $^{32}O_2$ in an aqueous solution.[43] Such an agreement indicates that an electron transfer from $C_{60}^{\bullet-}/\gamma$-CyD to O_2 undergoes via an outer-sphere pathway.

$O_2^{\bullet-}$ Formation via $^1O_2$. Singlet oxygen ($^1O_2$: $^1\Delta_g$) produced in an energy transfer from $^3C_{60}$* to O_2 in Scheme 3 may also be reduced by NADH to $O_2^{\bullet-}$.[44] Thus, the oxidation of NADH by $^1O_2$ as well as $^3C_{60}*/\gamma$-CyD is examined by determining the quantum yield (Φ) of the C_{60}/γ-CyD-photosensitized oxidation of NADH by O_2. The observed Φ values (Φ_{obs}) were determined from the disappearance of the absorption due to NADH at 380 nm at different concentrations of NADH and O_2 (see Experimental Section) and the results are listed in Table 1.

In general, $^1O_2$ is quenched by physical and chemical processes in which only the latter gives the actual products.[45,46] A laser flash photolysis study by Peters et al.[44] has revealed that $^1O_2$ is quenched by both the physical and electron-transfer processes in competition with the decay of $^1O_2$ to the ground state (the lifetime τ in H_2O is 4.2 μs)[46] as shown in Scheme 4. The physical quenching process gives the ground-state reactant

SCHEME 4



pair, NADH and O_2, whereas the electron transfer produces $NADH^{\bullet+}$ and $O_2^{\bullet-}$. The deprotonation of NADH is followed by the second electron transfer to O_2 to produce NAD^+ and $O_2^{\bullet-}$ with a rate constant of 1.9×10^9 M^{-1} s^{-1}.[47] The total quenching constant (k_q) and the electron-transfer quenching rate constant (k_{etq}) have been determined as 7.9×10^7 M^{-1} s^{-1} and 4.3×10^7 M^{-1} s^{-1}, respectively.[44]

By combining Scheme 3 and Scheme 4, the quantum yield (Φ) of the C_{60}/γ-CyD-photosensitized oxidation of NADH by O_2 is derived as given by eq 11 (see Supporting Information for the derivation). The first term corresponds to a direct photoinduced electron-transfer pathway to $^3C_{60}^*/\gamma$-CyD and the second term corresponds to a $^1O_2$ pathway. Because all the rate

$$\Phi = \frac{k_{et}\tau_T[\mathrm{NADH}]}{1 + k_{et}\tau_T[\mathrm{NADH}] + k_{EN}\tau_T[\mathrm{O_2}]}\left(\frac{k_p}{k_p + k_b}\right) + \frac{k_{etq}\tau[\mathrm{NADH}]}{1 + k_q\tau[\mathrm{NADH}]}\,\frac{k_{EN}\tau_T[\mathrm{O_2}]}{1 + k_{et}\tau_T[\mathrm{NADH}] + k_{EN}\tau_T[\mathrm{O_2}]} \quad (11)$$

constants in eq 11 are known or determined in this study, the Φ value can be calculated using eq 11. The calculated Φ values (Φ_{calc}) are listed in Table 1, where they agree well with the experimental values (Φ_{obs}). Such an agreement confirms the validity of Schemes 3 and 4. The Φ_{calc} values in Table 1 are those corresponding to a direct photoinduced electron-transfer pathway. As expected from Schemes 3 and 4, the photoinduced electron-transfer pathway becomes important with increasing the NADH concentration but the $^1O_2$ pathway (Scheme 4) dominates with increasing the O_2 concentration.

Actual Reactive Species for DNA Cleavage. As described above, the C_{60}/γ-CyD-photosensitized reduction of O_2 by NADH gives $O_2^{\bullet-}$ via both a direct photoinduced electron transfer from NADH to $^3C_{60}^*/\gamma$-CyD (Scheme 3) and an electron transfer from NADH to $^1O_2$ produced in an energy transfer from $^3C_{60}^*$ to O_2 (Scheme 4). Formation of $O_2^{\bullet-}$ may be inhibited by addition of NaN_3 which can quench $^3C_{60}^*/\gamma$-CyD in competition with an electron transfer from NADH and an energy transfer to O_2. In fact, $^3C_{60}^*/\gamma$-CyD is significantly quenched by NaN_3 as shown in Figure 9. The decay rate obeys pseudo-first-order kinetics and the pseudo-first-order decay rate constant of $^3C_{60}^*/\gamma$-CyD (k_{obs}) increases linearly with an increase in the concentration of NaN_3 (inset of Figure 9). From the slope of the linear plot is determined the quenching rate constant by NaN_3 as 1.7×10^8 M^{-1} s^{-1}.

This value is larger than the k_{et} value of an electron transfer from NADH to $^3C_{60}^*/\gamma$-CyD (1.0×10^8 M^{-1} s^{-1}), indicating that NaN_3, which is known as an efficient $^1O_2$ scavenger, can also act as a quencher of the triplet excited state $^3C_{60}^*/\gamma$-CyD itself. In such a case, photocleavage of DNA is also inhibited by adding sodium azide (NaN_3) as shown Figure 10 (lane 2 in reference to lane 1).

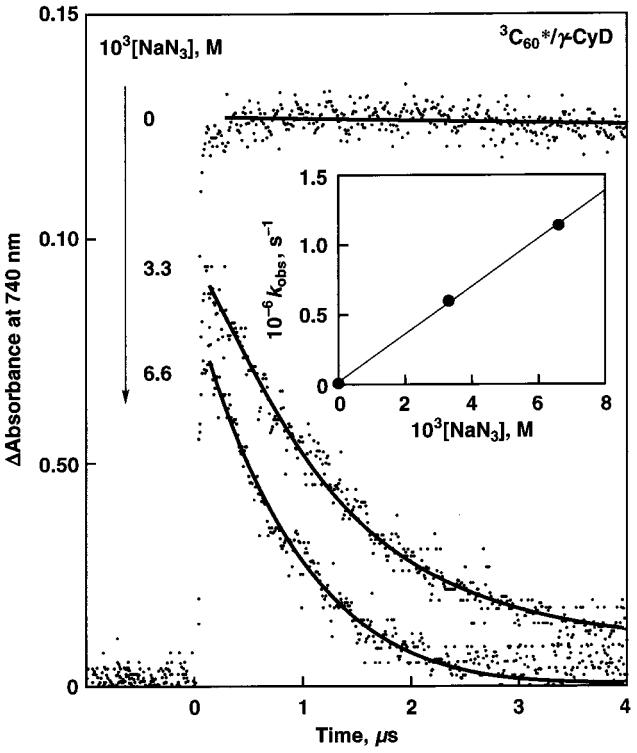


Figure 9. Time course changes of the absorbance at 740 nm due to $^3C_{60}^*/\gamma$-CyD (5.5×10^{-5} M) in the presence of different concentration of NaN_3 in phosphate buffer (50 mM, pH 7.4) at 295 K. $[NaN_3] = 0$, $0.3.3 \times 10^{-3}$, 6.6×10^{-3} M. Insets: plot of k_{obs} vs $[NaN_3]$.

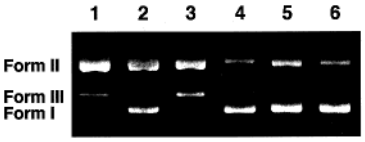
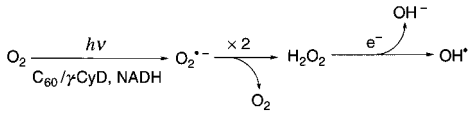


Figure 10. The effect of various additives on the DNA-cleaving activities of C_{60}/γ-CyD-NADH-O_2 system in phosphate buffer (50 mM, pH 7.4) after 1 h visible-light irradiation with 300-W reflector lamp at 273 K. Lane 1: C_{60}/γ-CyD (4.6×10^{-5} M) + NADH (1.0×10^{-2} M) (standard cleavage reaction). Lanes 2−6, standard reaction with additives: lane 2, with NaN_3 (1.0×10^{-2} M); lane 3, with SOD (100 μg mL^{-1}); lane 4, with catalase (100 μg mL^{-1}); lane 5, with methanol (1 M); lane 6, with ethanol (1 M).

SCHEME 5



Thus, formation of $O_2^{\bullet-}$ via $^3C_{60}^*/\gamma$-CyD may play an essential role in the DNA cleavage. However, this does not necessarily mean that $O_2^{\bullet-}$ is an actual reactive species for the DNA cleavage because $O_2^{\bullet-}$ is generally regarded as a rather unreactive radical species.[36] To examine the actual role of $O_2^{\bullet-}$ in the DNA cleavage, the DNA cleavage activity of the C_{60}/γ-CyD-NADH-O_2 system was determined in the presence of the enzyme superoxide dismutase (SOD). Addition of SOD shows no inhibitory effect on DNA cleavage as shown in Figure 10 (lane 3 in reference to lane 1). This indicates that H_2O_2 produced via disproportionation of $O_2^{\bullet-}$ is responsible for the DNA cleavage (Scheme 5). Such an SOD-dependent increase in DNA cleavage has been observed in other systems where $O_2^{\bullet-}$ is formed in the presence of electron donors.[48] Addition of the H_2O_2-destroying enzyme catalase significantly inhibits DNA cleavage (lane 4 in Figure 10). Addition of hydroxyl

radical (•OH) scavengers such as methanol (lane 5) and ethanol (lane 6) also inhibits DNA cleavage. The •OH is known as one of the most noxious reactive oxygen species, which induces the DNA cleavage.[49] The •OH can be produced in a trace-metal-dependent Fenton reaction of H_2O_2 (Scheme 5) as reported for the DNA cleavage by the antitumor antibiotic leinamycin.[50] Thus, any reagent that is capable of reducing oxygen to superoxide anion can produce hydroxyl radical.[49d] Thus, the actual reactive species for the photoinduced DNA cleavage in the aqueous C_{60}/γ-CyD-NADH-O_2 system may be hydroxyl radical as shown in Scheme 5.

In conclusion, the C_{60}/γ-CyD-photosensitized reduction of O_2 by NADH produces $O_2^{\bullet-}$ efficiently via a direct photoinduced electron transfer from NADH to $^3C_{60}$*/γ-CyD (Scheme 3) and via an electron transfer from NADH to $^1O_2$ produced by an energy transfer from $^3C_{60}$*/γ-CyD to O_2 (Scheme 4). The contribution of the former process increases with increasing the NADH concentration and the latter dominates with increasing the O_2 concentration. The $O_2^{\bullet-}$ thus produced gives H_2O_2, ultimately yielding hydroxyl radical (Scheme 5) which is an actual DNA-cleaving reagent.

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Supporting Information Available: Dose-dependences of C_{60}/γ-CyD and NADH on DNA cleavage (Figure S1) and derivation of eq 11 (2 pages). This material is available free of charge via the Internet at http://pubs.acs.org.

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