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Respiratory Chain Inhibition by Fullerene Derivatives: Hydrogen Peroxide Production Caused by Fullerene Derivatives and a Respiratory Chain System

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Abstract—Fullerene is a new type of carbon allotrope. We have shown that the fullerene derivative C_{60}-bis(*N,N*-dimethylpyrrolidinium iodide), a regio isomer mixture, inhibited *Escherichia coli* growth and dioxygen uptake caused by *E. coli* and glucose. This result indicates that the mechanism of the bacteriostatic effect is the inhibition of energy metabolism. In this study, we isolated two regio isomers of C_{60}-bis(*N,N*-dimethylpyrrolidinium iodide) and studied their effect on *E. coli* growth and on respiratory chain activity. In dioxygen uptake caused by the inner-membrane and NADH, the effect of fullerene derivatives was biphasic. At low concentrations of both fullerene derivatives, dioxygen uptake was inhibited, whereas at high concentrations, it was increased. At high concentrations, consumed dioxygen was converted to H_2O_2. An electrochemical study revealed that reduced fullerene derivatives react with dioxygen. This activity was closely related to a redox property of the isomers.

Fullerene, a condensed aromatic ring compound with an extended π conjugated system, is a new type of carbon allotrope that was discovered in 1985.[1] It has a unique cage-like shape, and a great deal of attention has been focused on its properties. Several years of extensive study of fullerene and its analogues have revealed many aspects of their physical properties and chemical reactivities.

The biological effects of fullerene and its derivatives are also important.[2] Some biological activities based on their unique physical properties and chemical reactivities have been reported. For example, DNA scissions[3,4] and oxidation of biological materials[5,6] depend on photo-induced active oxygen production by fullerene,[7,8] and enzyme-inhibition activities depend on the high hydrophobicity of fullerene.[9,10] We[11,12] and other groups[13,14] have reported antioxidant activities of fullerene, which are thought to depend on high reactivity for radicals. Other interesting biological effects of fullerene derivatives have also been reported.[15,16] Recently, much attention has been focused on the antioxidant activity of carboxy fullerene.

In contrast to carboxy fullerene, a bacteriostatic effect of cationic fullerene derivatives was found by Bosi[17] and by us.[18] We have also shown that a cationic fullerene derivative, C_{60}-bis(*N,N*-dimethylpyrrolidinium iodide), a regio isomer mixture, inhibited the dioxygen uptake caused by *Escherichia coli* and glucose.[18] This result indicates that the mechanism of the bacteriostatic effect is the inhibition of energy metabolism. However, as reported previously, the C_{60}-bis(*N,N*-dimethylpyrrolidinium iodide) used was a mixture of regio isomers.[18] Fullerene is a redox-active compound since it has a low LUMO level and a high HOMO level. Redox-active compounds often affect the biological electron transport system.

In this study, we isolated two main regio isomers of C_{60}-bis(*N,N*-dimethylpyrrolidinium iodide) (Fig. 1) and studied their effect on *E. coli* growth and respiratory chain activity. C_{60}-bis(*N,N*-dimethylpyrrolidinium iodide) inhibited *E. coli* growth and respiratory chain

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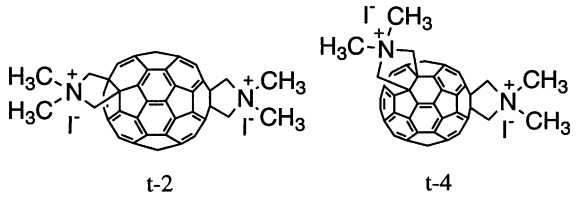


Figure 1. Structure of t-2 and t-4 isomers of C_{60}-bis(*N,N*-dimethylpyrrolidinium iodide).

activity. We found the active oxygen, H_2O_2, production activity of the fullerene derivatives from a respiratory chain system. We also found that a reduced form of the fullerene derivatives reacted with dioxygen. These activities were closely related to a redox property of fullerene derivatives.

Results

Preparation of the regioisomers of C_{60}-bis(*N,N*-dimethylpyrrolidinium iodide)

According to the procedure described by Lu et al.,[19] t-2 and t-4 C_{60}-bis(*N*-methylpyrrolidine) was prepared. Both t-2 and t-4 are main isomers of C_{60}-bis(*N*-methylpyrrolidine). Then both isomers were treated with methyl iodide to give a corresponding isomer of C_{60}-bis(*N,N*-dimethylpyrrolidinium iodide).

E. coli growth inhibition effect of the isomers

Figure 2 shows the effect of t-2 and t-4 isomers on *E. coli* growth. Only 1 μM of t-2 and t-4 inhibited growth completely, but the growth inhibition effect of t-2 was stronger than that of t-4.

Effect of fullerene derivatives on dioxygen uptake caused by *E. coli* respiratory chain

We previously found that C_{60}-bis(*N,N*-dimethylpyrrolidinium iodide) (mixture of regio isomers) suppressed energy metabolism in *E. coli*.[18] Subsequently, an effect of the fullerene derivatives on respiratory chain activity was investigated.

An *E. coli* inner-membrane fraction was prepared according to Kita et al.[20] Respiratory chain activity was determined by the initial dioxygen uptake rate caused by the inner-membrane fraction and NADH.

The effect of fullerene derivatives on the dioxygen uptake was biphasic. An addition of the fullerene derivatives (up to 5 μM) decreased the dioxygen uptake rate. Further addition of fullerenes (more than 10 μM) gradually increased the uptake rate but it was still slower than the control level. At 2.5 μM, the t-2 isomer was more effective in inhibiting dioxygen uptake than t-4, but from 5 μM to 12.5 μM, t-4 was an effective inhibitor. At high concentrations, dioxygen uptake rate was accelerated. The dioxygen uptake was more greatly enhanced by t-4 than by t-2 (Fig. 3). When the reaction was done in the dark, the effect of 25 μM of t-4 did not change (data not shown). Without the inner-membrane, 50 μM of t-4 caused light-dependent dioxygen consumption in the presence of NADH, but its rate was very slow (less than 3 μM/min).

Effect of catalase on dioxygen uptake

To elucidate a mechanism of dioxygen uptake enhancement, an effect of a catalase was investigated. The addition of catalase to the reaction mixture prior to the

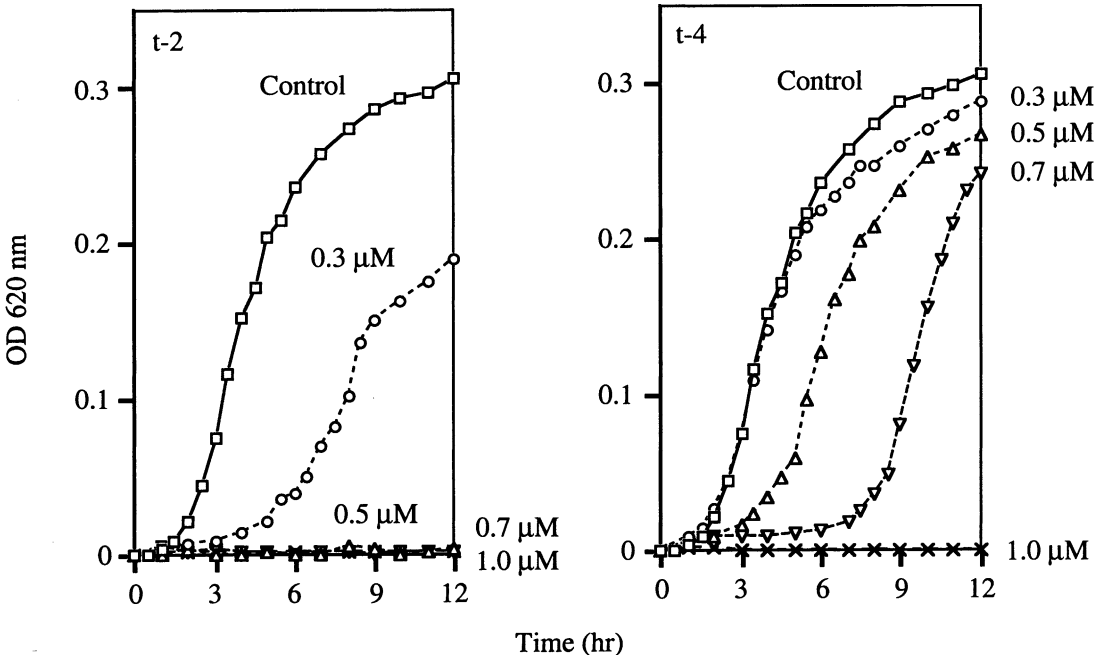


Figure 2. Effect of fullerene derivatives on *E. coli* growth. The culture medium contained $MgSO_4 \cdot 7H_2O$ 0.2 g, citric acid·H_2O 2.0 g, K_2HPO_4 10.0 g, $NaNH_4HPO_4 \cdot 4H_2O$ 3.5 g, vitamin B_{12} 1.0 mg, and glucose 5.0 g in 1 L. *E. coli* was cultured at 37 °C. Growth was monitored in terms of changes in turbidity at 630 nm using the tube with a photoelectric colorimeter. Values are means of two experiments.

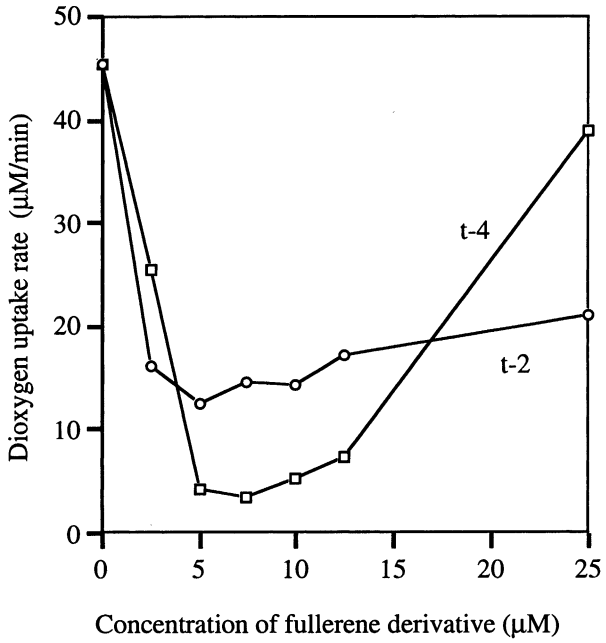


Figure 3. Effect of fullerene derivatives on dioxygen uptake rate caused by an *E. coli* inner-membrane fraction and NADH. The reaction solution contained 0.4 mM NADH and 0.1 mM EDTA in 50 mM potassium phosphate at pH 7.8. The reaction was started by the addition of an *E. coli* inner-membrane fraction (17 μg protein/mL) at 37 °C. Values are means of two experiments and the variability of this experiment was less than 5%.

reaction (12.5 μM of t-2) reduced the dioxygen uptake rate by half, whereas the effect of the catalase was small in t-4 at 12.5 μM (Table 1). The catalase did not affect the dioxygen uptake rate in the absence of fullerene derivatives. When H_2O_2 is produced from dioxygen, the catalase decreases the dioxygen uptake rate by half because the catalase returns half the amount of H_2O_2 to O_2 (eq 1). That is, the catalase produces half the amount of dioxygen from H_2O_2. In the case of t-2, dioxygen was reduced to produce H_2O_2, not H_2O. Table 1 shows the H_2O_2 and H_2O production rates calculated by the catalase effect. The H_2O production rate was calculated by subtracting the H_2O_2 production rate from the O_2 uptake rate. The H_2O production rate represents respiratory chain activity. The t-2 isomer inhibited respiratory chain activity almost completely at 12.5 μM, but t-4 did not inhibit it completely.

$$2H_2O_2 \rightarrow O_2 + 2H_2O \quad (1)$$

Reaction of the reduced form of the fullerene derivatives with dioxygen

Next, we examined the reaction of reduced fullerene derivatives with dioxygen by the electrochemical method. Figure 4 shows the cyclic voltammograms of t-2 and t-4 isomers. The reduction potential (vs. Ag/AgCl) was −710 mV (t-2, Fig. 4A) and −780 mV (t-4, Fig. 4C). Under anaerobic conditions, t-2 was reversibly reducible, and the re-oxidation wave was slightly diminished under 100% dioxygen (Fig. 4A and B). t-4 was almost reversibly reducible under N_2, and the re-oxidation wave was completely diminished under only 20% dioxygen (under air) (Fig. 4C and D). These results show, first, that t-2 is more easily reduced than t-4, and second, that the reduced forms of both derivatives react with dioxygen and this reaction was faster in the case of t-4.

Discussion

The effect of fullerene derivatives on dioxygen uptake caused by *E. coli* inner-membrane and NADH was biphasic. At low concentrations of the isomer, both derivatives inhibited dioxygen uptake, whereas at high concentrations, both increased it (Fig. 3). However, at high concentrations of fullerene derivatives, consumed dioxygen was converted to active oxygen, H_2O_2. In spite of dioxygen uptake enhancement by a high concentration of the fullerene derivatives, respiratory chain activity was not stimulated but was inhibited.

At 12.5 μM, the t-2 isomer inhibited respiratory chain activity completely and produced active oxygen, H_2O_2, more than t-4, but the t-4 isomer was effective in dioxygen uptake enhancement at 25 μM. These results were consistent with those of the electrochemical experiment; that is, the t-2 isomer was more easily reduced than t-4, but the reduced form of t-4 reacted with dioxygen faster than that of t-2. At low concentrations of the fullerene derivatives, the reduction of fullerene (Scheme 1, Step 1) might be a rate-limiting step in active oxygen production, so t-2 was more effective in active oxygen production and respiratory chain inhibition. However, at high concentrations, the reduction of the fullerene derivative becomes faster, and the reaction of the reduced fullerene with dioxygen (Scheme 1, Step 2) might be limiting.

Table 1. Effect of catalase on dioxygen uptake rate

	Dioxygen uptake rate (μM/min)	H_2O_2 production rate (μM/min)	H_2O production rate (μM/min)
Complete (no fullerene derivative)	38.3	0	38.3×2
+ catalase	38.8		
+ t-2 (12.5 μM)	23.1	21.4	1.7×2
+ t-2 (12.5 μM) + catalase	12.4		
+ t-4 (12.5 μM)	10.1	3.8	6.3×2
+ t-4 (12.5 μM) + catalase	8.2		

Complete contained 0.4 mM NADH and 0.1 mM EDTA in 50 mM potassium phosphate at pH 7.8. Concentration of catalase was 1500 U/mL. The reaction was started by the addition of an *E. coli* inner-membrane fraction (17 μg protein/mL) at 37 °C. The H_2O_2 production rate was calculated by following equation; 2×[(O_2 uptake rate in the absence of catalase)−(O_2 uptake rate in the presence of catalase)]. The H_2O production rate was calculated by subtracting the H_2O_2 production rate from the O_2 uptake rate. Values are means of two experiments.

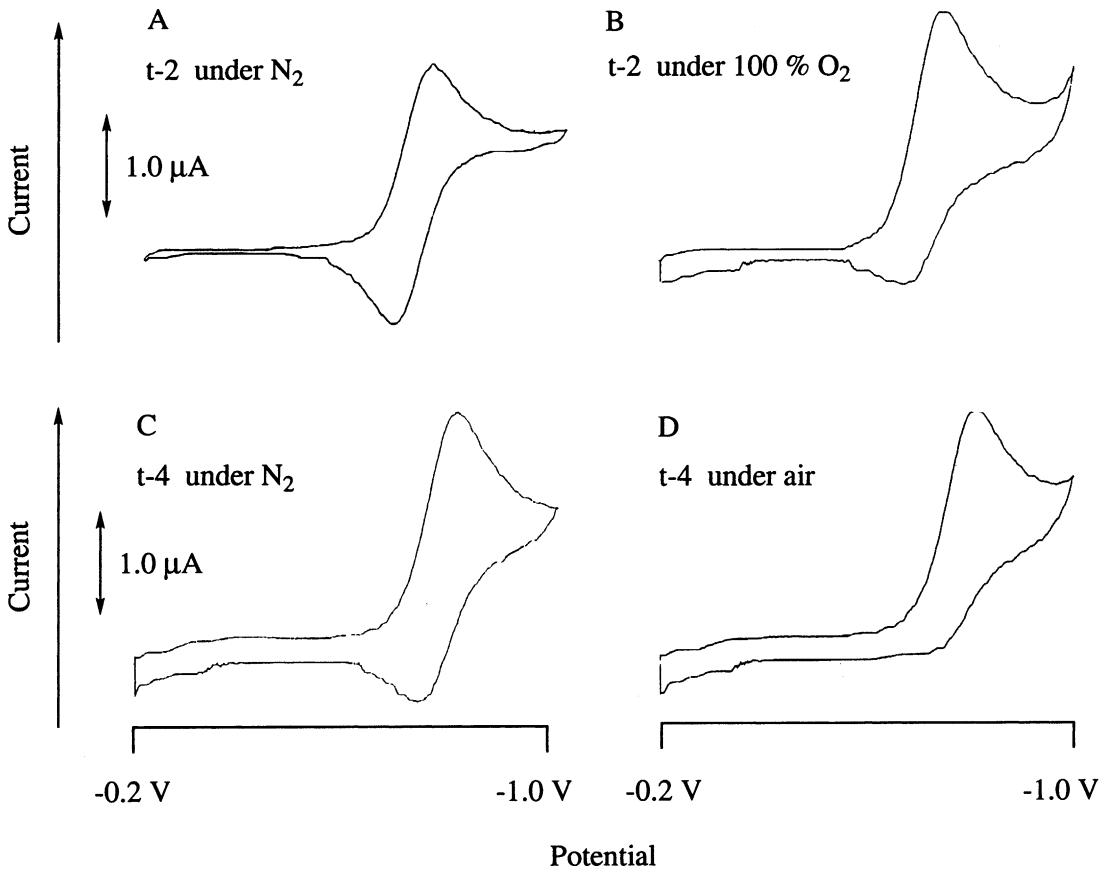
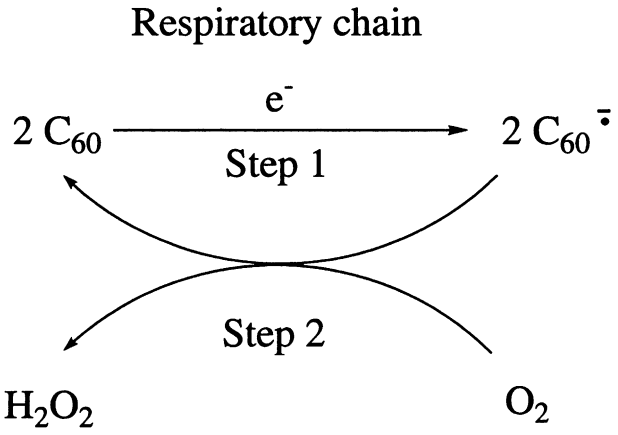


Figure 4. Cyclic voltammograms of t-2 and t-4 isomers. Reaction mixture contains 0.1 M $(n\text{-Bu})_4NPF_6$ and 1.0 mM fullerene derivatives in DMF.



Scheme 1. Active oxygen formation.

In contrast to dioxygen uptake enhancement, the t-2 isomer was effective in the inhibition of the respiratory chain. At 12.5 μM, t-2 inhibited the respiratory chain activity almost completely, whereas t-4 did not completely inhibit it. t-2 more effectively suppressed *E. coli* growth than t-4. Both results indicate that the growth inhibition mechanism is respiratory chain inhibition. The electrochemical experiment shows that the t-2 isomer was more easily reduced than the t-4 isomer. It is thought that t-2 was reduced by the respiratory chain system from a low concentration and then inhibited respiratory chain activity at low concentrations. The growth inhibition was more sensitive than the O_2 uptake inhibition. This may be explained by the accumulation of the fullerenes within the cells or within the membrane.

We assume that an inhibition site in the respiratory chain is flovoenzyme because fullerene derivatives inhibit flavoenzymes such as glutathione reductase.[21] This assumption needs to be investigated in future studies.

There have been many papers on photo-induced singlet oxygen production mediated by fullerene and its derivatives.[3–8] Recently, Yamakoshi et al. have reported that photo-irradiation promotes the electron transfer from NADH to C_{60} and the C_{60} anion radical reduces dioxygen to give superoxide.[22] These are non-enzymatic and photo-irradiation-dependent active oxygen productions. Indeed, dioxygen was consumed in the presence of 50 μM of t-4, NADH, and light (without the inner-membrane), but the rate was very slow (less than 3 μM/min in our conditions). We also investigated an effect of light on dioxygen uptake enhancement caused by a high concentration of fullerene derivative and the inner-membrane system and found no effect. These data indicated that the electron transfer from respiratory chain to fullerene derivative was not depending on light.

This paper is the first report that fullerene derivatives produce active oxygen in biological system without photo-irradiation.

We are now investigating antibacterial and anticancer activities of the fullerene derivatives.

Experimental

Materials

Reagents were all reagent-grade commercial products. C_{60} was obtained from MRT Ltd., D-glucose, vitamin B_{12}, and catalase were obtained from Sigma Chemical Co. NADH was from the Oriental Yeast Co.

Preparation of C_{60}-bis(*N,N*-dimethylpyrrolidinium iodide) (t-2)

t-2 isomer of C_{60}-bis(*N*-methylpyrrolidine) (70.3 mg, 84.3 μmol) was dissolved in methyl iodide (15 mL) and stirred for 72 h at room temperature to give a brown precipitate. The precipitate was collected by filtration, then washed with toluene, benzene, and dichloromethane to afford a red-brownish powder, t-2 isomer of C_{60}-bis(*N,N*-dimethylpyrrolidinium iodide) (86.4 mg, yield 91.7%). Product identification was done by ^{1}H NMR and high resolution FAB MS. δ ^{1}H (500 MHz, DMSO-d_6) 6.01 (d, $J=12.7$ Hz, 2H, $-CH_2-$), 5.82 (d, $J=12.7$ Hz, 2H, $-CH_2-$), 5.71 (d, $J=9.9$ Hz, 2H, $-CH_2-$), 5.67 (d, $J=9.9$ Hz, 2H, $-CH_2-$), 4.25 (s, 6H, $-CH_3$), 4.03 (s, 6H, $-CH_3$); high resolution FAB MS, found ($C_{68}H_{20}N_2I_2$) 865.1685 ($M^+ + 1\text{-}2I^-$, err. −2.0 mmu).

As we previously reported,[12] the ^{1}H NMR signal of *N*–CH_3 was shifted and separated into two signals after methylation.

Preparation of C_{60}-bis(*N,N*-dimethylpyrrolidinium iodide) (t-4)

T-4 isomer of C_{60}-bis(*N,N*-dimethylpyrrolidinium iodide) was prepared by the same method as described above. Starting from t-4 isomer of C_{60}-bis(*N*-methylpyrrolidine) (91.2 mg, 109 μmol), the title compound was yielded as brawn powder (87.7 mg, yield 72.0%). δ ^{1}H (500 MHz, DMSO-d_6), 5.7–5.4 (m, 8H, $-CH_2-$), 3.99 (s, 6H, $-CH_3$), 3.87 (s, 6H, $-CH_3$); high resolution FAB MS, found ($C_{68}H_{20}N_2I_2$) 865.1685 ($M^+ + 1\text{-}2I^-$, err. −2.0 mmu).

E. coli growth inhibition

E. coli B B_{12}^-, ATCC 29682, was used throughout the process. *E. coli* was cultured at 37 °C in a water bath shaker at 140 rpm with a glass tube (diameter = 15 mm) to a culture volume ratio of 5:1. Growth was monitored in terms of changes in turbidity at 630 nm using the tube with a photoelectric colorimeter. The culture medium contained $MgSO_4 \cdot 7H_2O$ 0.2 g, citric acid·H_2O 2.0 g, K_2HPO_4 10.0 g, $NaNH_4HPO_4 \cdot 4H_2O$ 3.5 g, vitamin B_{12} 1.0 mg, and glucose 5.0 g in 1 L. Fullerene derivatives were dissolved in DMSO and then added to the medium. The final DMSO concentration was less than 1.0 and 1.0% DMSO did not affect *E. coli* growth.

Respiratory chain activity

The *E. coli* inner-membrane fraction was prepared according to Kita et al.[18] The dioxygen uptake was monitored polarographically with a Clark-type electrode. The reaction solution contained 0.4 mM NADH and 0.1 mM EDTA in 50 mM potassium phosphate at pH 7.8. The reaction was started by the addition of an *E. coli* inner-membrane fraction (17 μg protein/mL) at 37 °C. Fullerene derivatives were also dissolved in DMSO and then added to the reaction buffer. The final DMSO concentration was less than 1.0, and 1.0% DMSO did not affect dioxygen consumption. Values in Figure 3 are means of two experiments. Using the same inner-membrane preparation, the variability of this experiment was less than 5%.

Electrochemical measurement

DMF was distilled under reduced pressure prior to use. All measurements were performed at an ambient temperature in a 0.1 M DMF solution of $(n\text{-}Bu)_4NPF_6$ at a scan speed of 50 mV/s. The concentration of C_{60} derivatives was 1.0 mM. A platinum wire was used as a working electrode, and a platinum wire was used as the counter electrode. The reference electrode was Ag/AgCl.

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