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SUPEROXIDE RADICAL QUENCHING AND CYTOCHROME *C* PEROXIDASE-LIKE ACTIVITY OF C_{60}-DIMALONIC ACID, $C_{62}(COOH)_4$

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Abstract: A water-soluble fullerene derivative (C_{60}-dimalonic acid, $C_{62}(COOH)_4$) quenches superoxide radical ($O_2^{\cdot -}$), in the xanthine/xanthine oxidase system, and oxidizes reduced cyt. *c* in the presence of hydrogen peroxide.

Fullerene and its analogues have been widely explored for several years, and many aspects of their chemical reactivity have been revealed. However, little is known about their biological activities,[1-3] mainly because of the poor solubility of the compounds in aqueous media. We have been investigating their metabolic reactions[4] and the biological activities of fullerene, its analogues, and their metabolites.

Active oxygen species such as superoxide radical ($O_2^{\cdot -}$), ROOH, and OH radical, produced during oxidative stress, *etc*, cause various kinds of biological damage. Redox-active compounds often affect the production and/or decomposition of active oxygen species in biological systems.[5, 6] Fullerene is a redox-active compound since it has a low LUMO level and a high HOMO level. We therefore examined the effect of a water-soluble fullerene derivative on cytochrome *c* (cyt. *c*) reduction in the xanthine/xanthine oxidase system, and found $O_2^{\cdot -}$ radical-quenching activity. We also observed cyt. *c* peroxidase-like activity of the fullerene derivative.

Recently, a water-soluble polyhydroxylated C_{60} derivative (fullerenol) was shown to have quenching activity for $O_2^{\cdot -}$.[7] However, the properties of fullerenol are quite different from those of the parent C_{60}, since conjugated double bonds of C_{60} are widely broken in fullerenol. Our interest is in the fullerene moiety itself. We therefore selected C_{60}-dimalonic acid ($C_{62}(COOH)_4$, Fig. 1), which has sufficient water solubility and is thought to have rather similar properties to C_{60}. $C_{62}(COOEt)_4$ was synthesized and its "equatorial" form was purified,[8] then converted to $C_{62}(COOH)_4$.[9-11] We examined the effect of this compound in the xanthine/xanthine oxidase system.[12] The production of $O_2^{\cdot -}$ was measured in term of cyt. *c* reduction determined from the increase in absorbance at 550 nm.

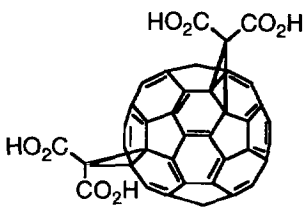

Fig. 1 "equatorial"-C_{60}-dimalonic acid $C_{62}(COOH)_4$.

Fig. 2 shows the effect of $C_{62}(COOH)_4$ on cyt. *c* reduction in the xanthine/xanthine oxidase system. The complete system (A in Fig. 2) contained cyt. *c* (10 μM) and xanthine (50 μM) in buffer solution (50 mM potassium phosphate buffer pH 7.8, containing 0.1 mM ethylenediaminetetraacetic acid) at 25 °C. When xanthine oxidase was added to the solution to generate $O_2^{\cdot -}$, cyt. *c* was reduced and the absorbance at 550 nm increased. The increase stopped at around 10 minutes after the addition of xanthine oxidase because cyt. *c* was completely reduced. When $C_{62}(COOH)_4$ (50 μM) was added to the complete system (B in Fig. 2), the initial rate of increase of the absorbance at 550 nm was slowed down. The cyt. *c* reduction again stopped at around 10 minutes, and then the absorbance at 550 nm decreased slowly. The changes depended on the quantity of $C_{62}(COOH)_4$. To investigate the effect of $C_{62}(COOH)_4$ on xanthine oxidase activity, uric acid production was measured by HPLC. Formation of uric acid was not affected by the addition of $C_{62}(COOH)_4$ under this reaction condition (data not shown). The decrease of absorbance at 550 nm was completely inhibited by addition of catalase (2,400 U/ml) to the xanthine oxidase/$C_{62}(COOH)_4$ solution (C in Fig. 2). Under this condition, produced H_2O_2 was completely converted to H_2O and O_2 by catalase. These results indicated that $O_2^{\cdot -}$ was quenched by $C_{62}(COOH)_4$, and both H_2O_2 and $C_{62}(COOH)_4$ were essential for the oxidation of reduced cyt. *c*. This kind of oxidation is called as cyt. *c* peroxidase activity.

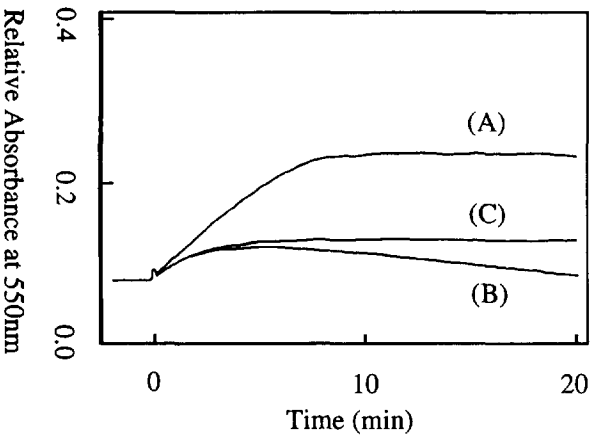

Fig. 2 The effect of $C_{62}(COOH)_4$ on cyt. *c* reduction by xanthine and xanthine oxidase. (A) : complete, (B) : (A) + $C_{62}(COOH)_4$ 50 μM, (C) : (A) + $C_{62}(COOH)_4$ 50 μM + catalase 2,400 U/ml.

Fig. 3 shows the spectral change of cyt. *c* upon addition of H_2O_2 and $C_{62}(COOH)_4$. The spectra of oxidized and reduced cyt. *c* (10 μM) are shown as A and B in Fig. 3, respectively. Addition of $C_{62}(COOH)_4$ (25 μM) to reduced cyt. *c* raised the base line of the spectrum, but oxidation of reduced cyt. *c* was very slow (C in Fig. 3). The addition of H_2O_2 (100 μM) to the solution removed reduced cyt. *c* completely within 5 minutes to give oxidized cyt. *c* (D in Fig. 3). In contrast, when H_2O_2 (100 μM) was added to reduced cyt. *c* , oxidation of it was very slow. These results confirm that $C_{62}(COOH)_4$ has cyt. *c* peroxidase-like activity.

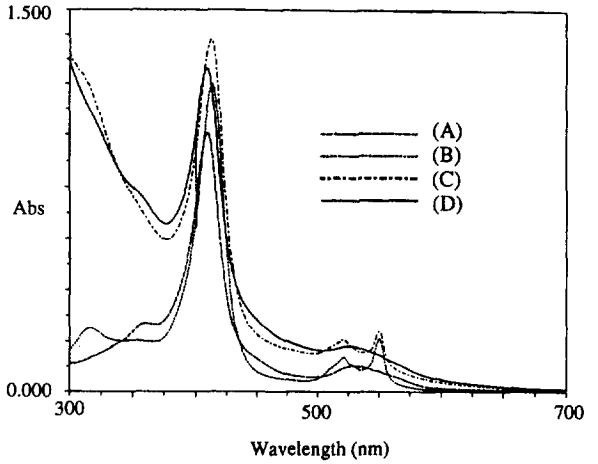

Fig. 3 Spectral change of cyt.*c* with $C_{62}(COOH)_4$ and H_2O_2.
(A) : oxidized cyt. *c*, (B) : reduced cyt. *c*, (C) : the spectum was mesaured 5 minutes after the addition of $C_{62}(COOH)_4$ 25 μM to (B), (D) : the spectrum was measured 5 minutes after the addition of H_2O_2 100 μM to (C).

$C_{62}(COOH)_4$ must have another activity in addition to $O_2^{\cdot -}$ quenching and cyt. *c* peroxidase-like activity, since, when $C_{62}(COOH)_4$ was added, the absorbance at 550 nm did not increase to the level of complete system even in the presence of catalase (C versus A in Fig. 2). The details are under investigation.

The generation of singlet oxygen ($^1O_2$) by irradiation of fullerene[13, 14] and its derivatives[15] is well known. But few studies[7] have been reported on the interaction of fullerene and active oxygen species other than $^1O_2$. In biological systems, the peroxidase activity of the fullerene derivative could enhance the toxicity of H_2O_2 or, in contrast, decomposition of H_2O_2 could be catalyzed by the fullerene derivative in the presence of a suitable electron donor. It is of interest to study the effects of fullerene derivatives on active oxygen toxicity.

C_{60} solubilized by polyvinylpyrrolidone[16] has similar activities, but its activities are weaker than those of $C_{62}(COOH)_4$ (data not shown), probably because of the encapsulation of C_{60} by polyvinylpyrrolidone.

In conclusion, we have shown that $C_{62}(COOH)_4$ has $O_2^{\cdot -}$ radical-quenching and cyt. *c* peroxidase-like activities. We are planning to investigate the reaction mechanisms in more detail and to elucidate the effects of the peroxidase-like activity of $C_{62}(COOH)_4$ on various biological compounds.

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8. $C_{62}(COOEt)_4$ ("equatorial" form) was purified by silica gel chromatography. Its NMR data showed that it contained its regioisomers less than 5%.

9. Further purification was not performed after hydrolysis of $C_{62}(COOEt)_4$.

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