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Photoinduced Reduction of Methylviologen by Ascorbate Using Chlorophyllin in Liposome System

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Photoindeced electron transport across bilayer lipid membrane was studied, using chlorophyllin (Chln), a water-soluble derivative of chlorophyll. Photoreduction of methylviologen (MV^{2+}) by ascorbate was observed only in the liposome system containing ascorbate in the internal aqueous phase and MV^{2+} in the external aqueous phase. This suggests asymmetry of bilayer lipid membrane. This reaction rate was not affected by the addition of carbonyl cyanide *m*-chlorophenylhydrazone in the system buffered by tris(hydroxymethyl)aminomethane (Tris), but strongly affected by *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid. In the system buffered by Tris, a proton carrier is not Chln having carboxyl groups but Tris possessing primary amine group. Replacement of the central Mg of Chln with Zn gives a higher rate of MV^{2+} photoreduction, but when Cu was used in place of Zn, photoreduction was not observed. The central metal may affect the redox potential and the life time of excited Chln.

Membrane-like structure plays an important role in bioenergetic systems. It is well-known that thylakoid membrane in chloroplast is indispensable to photosynthesis. There are three major functions in biological membrane. First, membrane is a barrier to separate the internal aqueous phase of cell from the external. Second, it can orient the functional units (enzyme, protein *etc.*) in the highly ordered forms. Third, it is a reaction medium of chemical and/or physical reactions. Consequently, thylakoid membrane enables active transport of electrons by interfering with back reaction and generates pH gradient to synthesize ATP.

There have been many reports on photoinduced electron transport across bilayer lipid membrane sensitized by chlorophyll,[1,2] amphiphilic Zn(II) porphyrins,[3,4] or Ru(II) complexes.[5,6] But these photosensitized redox reactions often occur effectively in homogeneous solutions.

We previously reported that photoinduced electron transport across bilayer lipid membrane can take place sensitized by chlorophyllin.[7] Chlorophyllin is a water-soluble derivative of chlorophyll, and a fairy green pigment which resembles chlorophyll in its photochemical reactivity.[8-10]

We report here that photoreduction of methylviologen by ascorbate using chlorophyllin as a sensitizer in liposome system. This reaction doesn't occur in homogeneous solution. Ascorbate is a reversible electron donor which is different from EDTA and triethanolamine as sacrificial electron donors.

Experimental

Materials. *Chlorophyllin* a: Chlorophyll a was extracted from fresh spinach leaves with cold acetone and purified according to the procedure of Omata and Murata.[11] Chlorophyllin a (Chln) was prepared from chlorophyll a by the method of Oster and his coworkers.[12]

Cu- and Zn-chlorophyllin a: Cu- and Zn-chlorophyll were prepared from their original chlorophyll using to the procedure of Onoue[13] and subsequently converted into Cu-chlorophyllin a (Cu-chln) and Zn-chlorophyllin a (Zn-chln).

Lecithin: Lecithin (phosphatidylcholine) was isolated from hen egg yolk and purified with the chromatographic procedure.[14]

Methylviologen (MV^{2+}) and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) were purchased from Sigma Chemical Co. and the other chemicals were reagent grade and used without further purification.

Liposome. Lecithin in chloroform was dissolved in benzene and colyophylized. Liposomes were prepared by sonicating lecithin in the solution buffered by 1 mol dm^{-3} Tris-Cl 0.1 mol dm^{-3} KCl (pH 7.5) under an N_2 atmosphere for 1 h at 4 °C. The dispersion was then centrifuged to remove metal fragments. External ions were separated from liposomes by gel-filtration on a 2×50 cm Sephadex G-50 column. The samples were purged of O_2 by bubbling a stream of Ar gas for 30 min at 4 °C.

Measurements. Photoreaction was carried out under an atmosphere of Ar gas sealed in the Pyrex tube with UV cell on the bottom. Light was irradiated with 500 W Xenon lamp (Ushio UXL-500D). Glass filters (Toshiba IRQ-25, OR-57) were used to isolate red light. Absorption spectra were measured on Hitachi spectrophotometer. Photoinduced electron transport was monitored by measuring the absorbance of reduced methylviologen ($MV^{+\cdot}$) at 605 nm.

Results and Discussions

Photoreduction of MV^{2+}. Transmembrane electron transport from ascorbate (Asc) to MV^{2+} sensitized by Chln was investigated in the various systems and the formation of $MV^{+\cdot}$ with time of irradiation as is shown in Fig. 1. In the homogeneous system, $MV^{+\cdot}$ was not observed. This is thought to be due to the rapid back reaction between oxidized ascorbate (DAH) and $MV^{+\cdot}$. In the liposome system containing MV^{2+} in the internal aqueous phase and Asc in the external, $MV^{+\cdot}$ didn't produce though the bilayer lipid membrane interfered with the back reaction. Photoreduc-

tion of MV^{2+} was observed only in the liposome system containing Asc in the internal aqueous phase and MV^{2+} in the external. The experimental results as described below were gained in this system.

The progressive spectral changes with time of irradiation are shown in Fig. 2. Chln in the liposome system showed a red absorption peak at 660 nm. However, on irradiation, a new absorption band appeared gradually at 690-nm as the 660 nm peak decreased. After the 690-nm peak was saturated, the absorption band due to $MV^{+\cdot}$ was observed to increase. From the previous experiment,[7,15] we confirmed the 690 nm absorbing species was an irreversible product of Chln (Chln'). In the present system, Chln didn't appear to sensitize MV^{2+} photoreduction but Chln' did and it was recycled because the spectrum after 20-min irradiation corresponded to that after 5-min irradiation when O_2 gas was added to the system to remove $MV^{+\cdot}$ absorption.

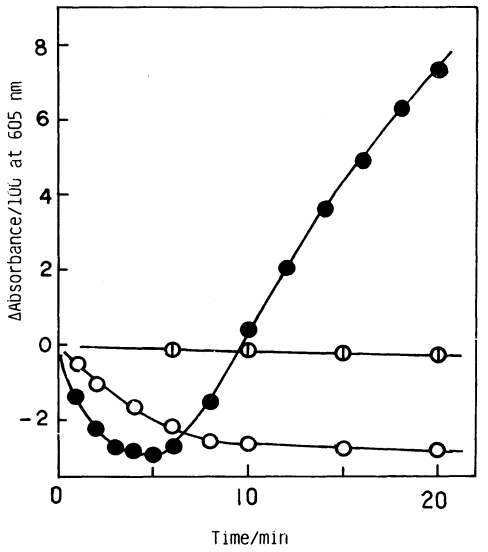

Fig. 1. Photoreduction of MV^{2+} in various systems. ⓘ: [Chln]=10 μmol dm^{-3}, [MV^{2+}]=10 mmol dm^{-3}, [AscNa]=10 mmol dm^{-3}, in Tris buffer, ○: [Chln]=10 μmol dm^{-3}, [MV^{2+}]=0.1 mol dm^{-3} (in), [AscNa]=10 mmol dm^{-3} (out), in liposome system, ●: [Chln]=10 μmol dm^{-3}, [AscNa]=1 mol dm^{-3} (in), [MV^{2+}]=10 mmol dm^{-3} (out) in liposome system.

The dependence of photoreduction rate of MV^{2+} on MV^{2+} concentration is shown in Fig. 3. The measurement of the concentration of $MV^{+\cdot}$ was based on the minimum absorbance at 605 nm as described above. The reduction rate increased rapidly with increasing the lower concentration region, and saturated at the higher concentration.

From these results, the reaction scheme is proposed and the difference of the reactivities of the two liposome systems is discussed. The reaction is thought to proceed as follows:

$$\text{Chln} \xrightarrow{h\nu} \text{Chln}' \quad (1)$$

$$\text{Chln}' \xrightarrow{h\nu} \text{Chln}'^{*} \quad (2)$$

$$\text{Chln}'^{*} + MV^{2+} \longrightarrow \text{Chln}'^{+\cdot} + MV^{+\cdot} \quad (3)$$

$$\text{Chln}'^{+\cdot} + \text{Asc}^{-} \longrightarrow \text{Chln}' + \text{DAH} \quad (4)$$

where Chln'* and Chln'$^{+\cdot}$ are excited Chln' and Chln' cation radical, respectively. The reaction (1) is the formation of the 690-nm absorbing species. The decrease of the absorbance at 605 nm at the initial step

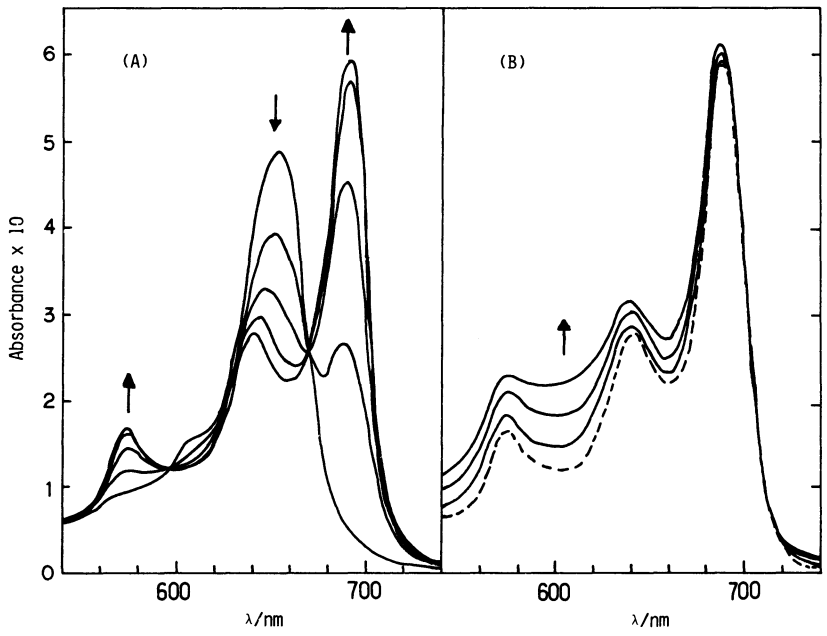

Fig. 2. Absorption spectra of liposome system.
(A) 0, 1, 2, 3, and 4 min after irradiation, (B) (——): 10, 15 and 20 min after irradiation and (----): bubbled O_2 after 20-min irradiation.

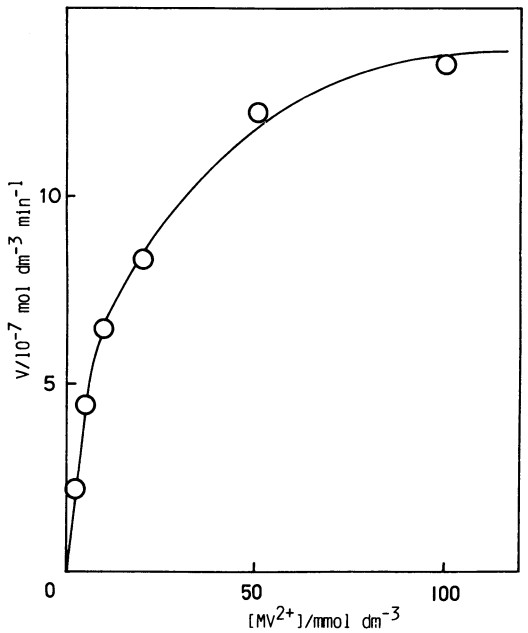

Fig. 3. Dependence of the reduction rate on MV^{2+} concentration.

was due to this reaction. The dependence of MV^{2+} photoreduction on MV^{2+} concentration is interpreted according to the scheme. In the lower MV^{2+} concentration, the reduction rate was determined by the reaction (3) and the observed rate was dependent on MV^{2+} concentration. On the other hand, as the reaction (3) was fast enough at the higher MV^{2+} concentration, the photoreduction rate was determined by the reaction (2). Therefore the observed rate was independent of MV^{2+} concentration.

The different results in the two liposome systems suggests asymmetry of bilayer lipid membrane. There have been several reports on asymmetry in sonicated liposomes.[16-20] The packing density of the inner-layer lipid molecules is more dense than that of the outer-layer lipid molecules owing to the difference of the curvature and causes the geometrical constraints to the head groups and the acyl chains of the inner-layer lipid molecules (Fig. 4[21]). This asymmetry facilitates the reaction (2) rather on the outer surface than on the inner surface. And another investigation indicates that the greater part of water-soluble Chln added to the liposome system exists on the outer surface.[15] Such a biased distribution of Chln is also more favorable to the reaction (2) on the outer surface than on the inner surface. Therefore, MV^{2+} photoreduction didn't occur in the liposome system containing MV^{2+}, in the internal and Asc in the external, but was observed in the liposome system containing Asc in the internal and MV^{2+} in the external.

Effect of CCCP. Chln was expected to play a role of a proton carrier as well as an electron carrier because Chln is tricarboxylic acid. The effect of CCCP was investigated in the liposome system buffered by

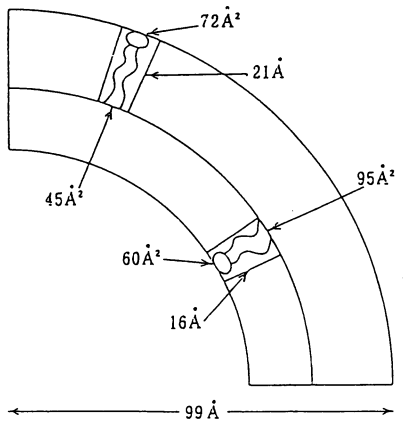

Fig. 4. Vesicle bilayer cross section showing the packing geometry of lipids within the inner and outer vesicle bilayer.

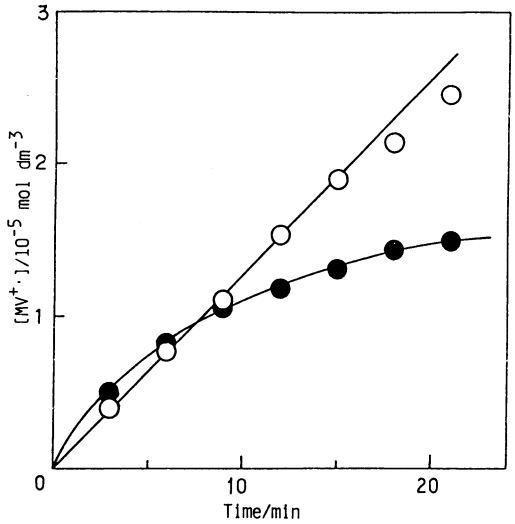

Fig. 5. Effect CCCP on MV^{2+} photoreduction in liposome system buffered by HEPES, pH 7.5.
●: without CCCP, ○: with 0.1 mmol dm^{-3} CCCP.

2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES), pH 7.5 because HEPES is impermeable to membrane.[22,23] The addition of CCCP strongly affected MV^{2+} photoreduction as is shown in Fig. 5. Contrary to our expectation, Chln didn't transport protons with electrons in the absence of CCCP and the formation of membrane potential suppressed the photoreduction. But in the system buffered by Tris, pH 7.5 the effect of CCCP on the reaction rate was not observed. Without CCCP, $MV^{+\cdot}$ production was nearly linear, not saturated for our measurement time. It was reported that Tris could penetrate membrane and that 'Hill reaction' was hindered at the high concentration of Tris because Tris, having the primary amine group, acted as an uncoupler.[23,24] From a consideration of these reports, it is consistent to conclude that Tris played a part of a proton carrier in our system since the concentration of Tris was 1 mol dm^{-1}.

Effect of Central Metal. The effect of the central

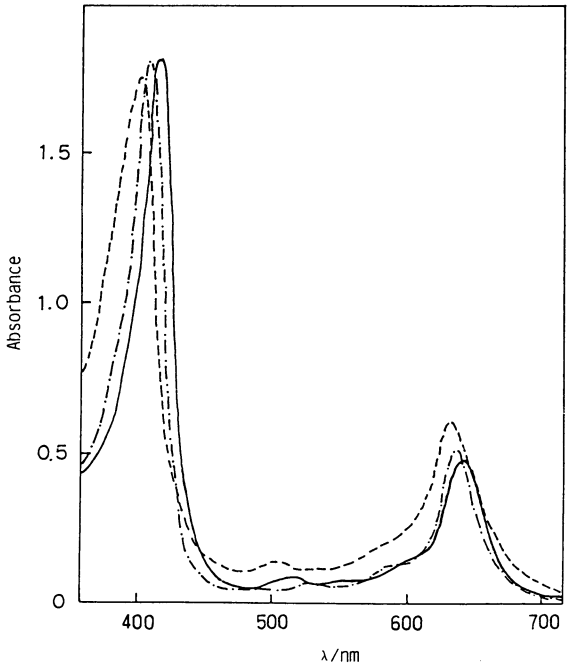

Fig. 6. Absorption spectra of chlorophyllin derivatives.
(——): Chln, (—·—): Zn-chln, (----): Cu-chln.

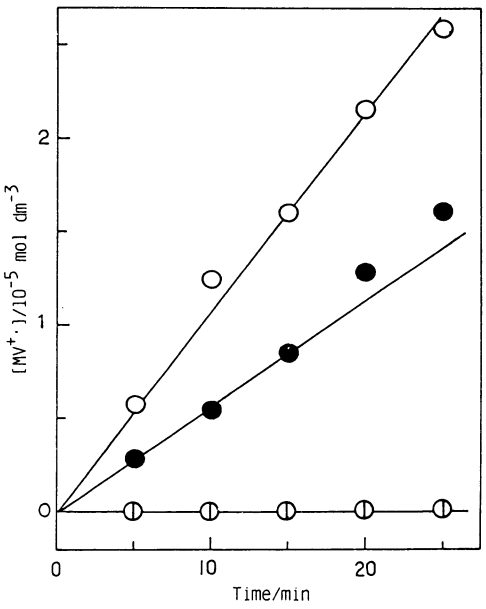

Fig. 7. Photoreduction of MV^{2+} sensitized by chlorophyllin derivatives.
●: Chln, ○: Zn-chln ⊕: Cu-chln.

metal of Chln on photoredox reaction was investigated by replacement of the central Mg with Cu or Zn. Though the spectra of these pigments bear much resemblance as is shown in Fig. 6, different photocatalytic reactivities were observed in MV^{2+} reduction as is shown in Fig. 7. The reactivity of pigment decreased in the order Zn-chln>Chln>Cu-chln=0. It is remarkable that Zn-chln reactivity was better than that of Chln which has the same Mg as chlorophyll, a natural photosynthetic pigment. The result indicates the redox potential and/or the life time of the excited state of Chln may depend on the central metal. The relative order of the redox potentials of these excited pigments may be Zn-chln>Chln>MV^{2+}>Cu-chln. Therefore Cu-chln couldn't sensitize the photoreduction of MV^{2+}.

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