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PHOTOINDUCED CHARGE SEPARATION IN LIPOSOMES CONTAINING CHLOROPHYLL *a*. II THE EFFECT OF ION TRANSPORT ACROSS MEMBRANE ON THE PHOTOREDUCTION OF $Fe(CN)_6^{3-}$

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Summary The reduction of $Fe(CN)_6^{3-}$ in the solution of outside liposome incorporating chlorophyll *a* was observed upon illumination with visible light under unaerobic condition. The photoreduction of $Fe(CN)_6^{3-}$ was enhanced by the presence of ion carrier, such as carbonylcyanide m-chlorophenylhydrazone, in the dispersion. The action spectrum of the photoreduction agreed with the absorption spectrum of chlorophyll *a* in liposome bilayer. The carrier-mediated proton transport was also observed upon illumination, occuring in the direction to compensate the excess charge produced by the photoinduced electron transport through the bilayer.

INTRODUCTION

Photochemical properties of pigments incorporated into phospholipid bilayer membrane have been extensively investigated for these years with the interests in the living photosynthetic systems and the light energy conversion. We reported that the photoreduction of Cu^{2+} in the solution outside liposome containing chlorophyll *a* was enhanced by a reductant, such as potassium ascorbate, localized in the solution inside liposome (1). The reduction rate will be affected by ion carriers across membrane because a charge unbalance between the outside and inside caused by the electron transfer will be readily compensated by the

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Abbreviations: Chlorophyll *a*, Chl; Phospholipid, PC
Carbonylcyanide m-chlorophenylhydrazone, CCCP
2,4-Dinitrophenol, DNP; Tetraphenylboron, TPB
Dimethyldibenzylammonium, DDA
Tris(hydroxymethyl)aminomethane, Tris

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carrier-mediated ion transport. In the present study, the effect of uncoupler, especially that of CCCP, on the photoreduction of $Fe(CN)_6^{3-}$ and the CCCP-mediated proton transport under illumination were described.

EXPERIMENTAL

Materials PC was extracted form hen egg yolk and purified by the method of Pangborn (2). Chl was extracted from fresh spinach leaves and purified by the method of Iriyama et al. (3). CCCP purchased from Aldrich, DNP and TPB from Wako Pure Chemicals Ltd., and DDA from Nakarai Chemicals Ltd. were all reagent grade and used without further purification.

Preparation of liposome dispersion PC colyophilized with Chl from benzene solution under vacuum was suspended in an Ar-saturated aqueous solution of a desired composition. The suspension was sonicated for 1 hr. at 20 kHz and 4 °C under Ar atmosphere followed by centrifugation at 17,000 g for 10 min. $K_3Fe(CN)_6$ and ion carrier were added to the resultant dispersion. The concentration of Chl in each sample solution was kept constant at 8×10^{-6} M, and Chl/PC molar ratio was at 1/400 in the present experiment.

Measurement of photoreduction of $Fe(CN)_6^{3-}$ Reduction of $Fe(CN)_6^{3-}$ was measured by using an ion selective electrode. The linearity between the electromotive force and logalithm of concentration of $Fe(CN)_6^{3-}$ was confirmed to hold in the concentration range from 10^{-6} to 10^{-3} M. The compositions of the external and the internal solutions were as follows; internal solution: 0.1 M KCl, 1.0 M TrisCl at pH 7.5, external solution: $K_3Fe(CN)_6$, 0.1 M KCl, 1.0 M Tris Cl at pH 7.5. A 20 ml glass cell with a flat glass window of 1.5 cm^2 was filled with the sample dispersion, and the dispersion was bubbled with Ar gas for 30 min. to remove dissolved oxygen, then the cell was sealed to prevent from oxygen penetrating to the sample. A sample was irradiated with a 500 W Xe lamp through glass filters (490-800 nm band pass) and 5 cm water layer. A set of interference filters was used as needed. The cell was blown with cool air during the illumination to prevent the sample from warming.

Measurement of photoinduced proton transport The composition of the solutions for the measurement of photoinduced pH change was as follows; internal solution: 0.1 M KCl, 0.01 M phosphate buffer at pH 7.5, external solution: $K_3Fe(CN)_6$, 0.1 M KCl. The composition of the external aqueous phase was changed by rapid passage of the dispersion over a column of Sephadex G-50. The pH change of the solution outside liposome was measured with a glass electrode.

RESULTS AND DISCUSSION

$Fe(CN)_6^{3-}$ in the external aqueous phase of liposome was reduced by an illumination with visible light, and the addition of CCCP, proton carrier, to the dispersion intensively facilitated the photoreduction. The action spectrum agreed with the absorption spectrum of Chl in the liposome dispersion, but the amount of bleached Chl was negligibly small compared with that of redued $Fe(CN)_6^{3-}$. Typical examples of the concentration change during the illumination are given in Figure 1. Using BLM incorporating Chl and separating two aqueous solutions of 0.1 M KCl and 0.01 M TrisCl at pH 7.0, photocurrent through the bilayer has been observed under illumination when 10^{-3} M $K_3Fe(CN)_6^{3-}$ was added in either solution (4). Tien has reported using similar systems, such as chloroplast extracts incorporating BLM in 0.1 M acetate buffer at pH 5, that photoinduced electromotive force across membrane was observed when $FeCl_3$ was added in one side of bathing solution (5). These results demonstrate that the photoreduction of $Fe(CN)_6^{3-}$ at one membrane-solution interface is coupled with the oxidation at the opposite interface through an electron transfer across the bilayer. In both cases of liposome and BLM, the presence of particular reductant in the opposite side to $Fe(CN)_6^{3-}$ was unnecessary suggesting the possibility of the oxidation of H_2O or OH^- (6).

To elucidate the effect of ion carriers the initial rate of $Fe(CN)_6^{3-}$ photoreduction and the final amount of reduced $Fe(CN)_6^{3-}$ are plotted against the concentration of CCCP in the dispersion in Figure 2. The reduction rate increased rapidly with increasing the concentration of CCCP in the lower concentration region, and saturated around 10^{-4} M of CCCP. At lower CCCP concentration the amount of reduced $Fe(CN)_6^{3-}$ was much greater than the amount of added CCCP, e.g. in the case of CCCP concentration of 10^{-5} M, the amount of reduced $Fe(CN)_6^{3-}$ was 9 x 10^{-5} M. This fact disprove the possibility that CCCP itself reacts with $Fe(CN)_6^{3-}$. It is supposed that the photoreduction of $Fe(CN)_6^{3-}$ was enhanced because the unbalance in the charge distribution between the exteranl and internal solutions of a liposome caused by the electron transfer

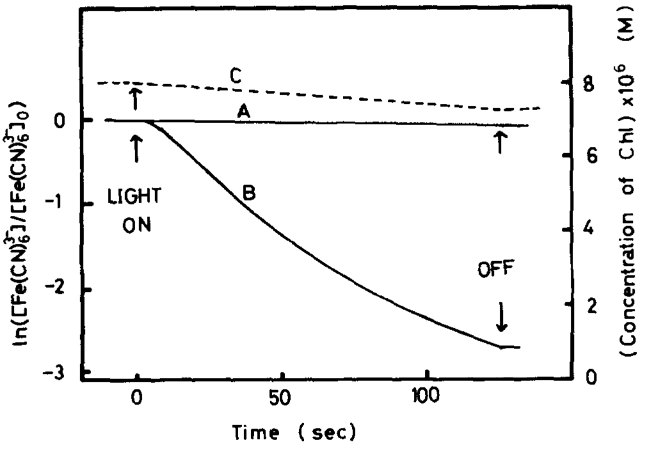

Figure 1. Time-course of $Fe(CN)_6^{3-}$ and Chl concentrations in the liposome dispersion upon illumination. $[Fe(CN)_6^{3-}]_0 = 2 \times 10^{-4}$ M. A; Time-course of $Fe(CN)_6^{3-}$ concentration in the absence of CCCP. B; the corresponding curve in the presence of 1.9×10^{-4} M CCCP. C; bleach of Chl in the case B.

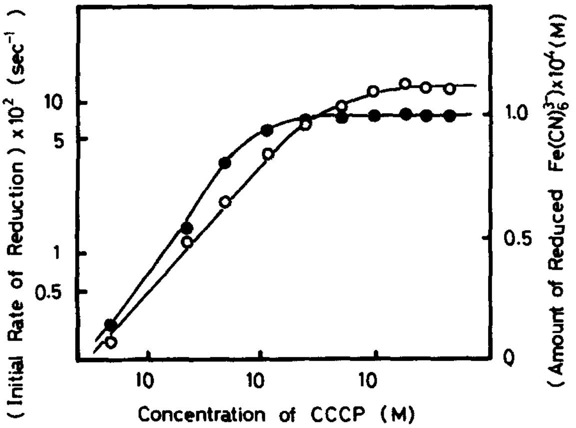

Figure 2. Plots of the initial reduction rate of $Fe(CN)_6^{3-}$ and the final amount of reduced $Fe(CN)_6^{3-}$ against the concentration of CCCP in the dispersion, at the initial $Fe(CN)_6^{3-}$ concentration of 1.0×10^{-4} M.

was compensated by the CCCP-mediated proton transport. In fact, the CCCP-mediated proton transport upon illumination was observed in a similar system. Figure 3 shows the pH change in the unbuffered external solution of liposome in the presence and absence of CCCP. It is of interest that a significant increase in the

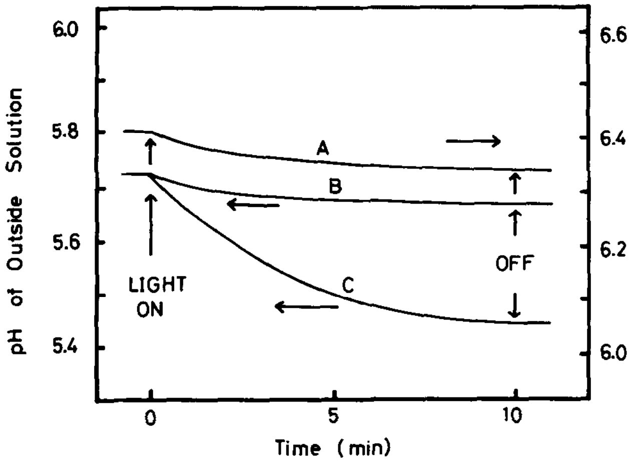

Figure 3. Change of pH in the external solution during illumination. The initial concentration of $Fe(CN)_6^{3-}$ is 10^{-4} M, and the CCCP concentration in the dispersion is 7 x 10^{-5} M. A; without CCCP and $Fe(CN)_6^{3-}$. B; with CCCP and without $Fe(CN)_6^{3-}$. C; with CCCP and $Fe(CN)_6^{3-}$.

proton concentration at the smaller pH side of the membrane was observed during illumination in the presence of CCCP. These results are qualitatively consistent with the results of the CCCP dependent photoreduction of $Fe(CN)_6^{3-}$, strongly suggesting that the increase in the proton concentration of the external phase is due to the proton transport from the internal to the external aqueous phase.

In addition, effects of other uncouplers on the reaction rate were examined for the case where the photoreduction occurred in the outside solution. Proton carrier, DNP and lipid soluble anion, TPB also facilitated the photoreduction of $Fe(CN)_6^{3-}$, whereas lipid soluble cation DDA did not when the concentration of DDA was 4.4 x 10^{-4} M. The latter may be explained by the fact that the total amount of cation in the internal phase is too small to compensate the unbalanced charge since the volume ratio of the external aqueous phase to the total internal aqueous phase of liposomes was 1,000 in this case.

Under the similar conditions Figure 1, a photoinduced formation of Chl cation radical was also observed by means of ESR (JEOL, JES-PE 3X), both in the absence and presence of CCCP. The ESR signal with peak to peak line width of 7.5 gauss nad g-value of 2.0028 was similar to the reported Chl cation radical (7). The signal intensity was saturated by the illumination for about 30 sec.

Taking these results into consideration, the following reaction scheme might be proposed when CCCP was used as an ion-carrier.

$\mathrm{Chl} \underset{}{\overset{h\nu}{\rightleftarrows}} \mathrm{Chl}^*$	k_1 k_{-1}	photoexcitation deactivation	(1)
$\mathrm{Chl}_o^* + \mathrm{Fe(CN)}_6^{3-} \longrightarrow \mathrm{Chl}_o^+ + \mathrm{Fe(CN)}_6^{4-}$	k_2	Chl cation radical formation	(2)
$\mathrm{Chl}_o^+ + \mathrm{Fe(CN)}_6^{4-} \longrightarrow \mathrm{Chl}_o + \mathrm{Fe(CN)}_6^{3-}$	k_3	back reaction	(3)
$\mathrm{Chl}_o^+ + \mathrm{Red}_i + \mathrm{H}_i^+ \longrightarrow \mathrm{Chl}_o + \mathrm{Ox}_i + \mathrm{H}_o^+$	k_4	electron transfer and proton transport	(4)

Where the subscripts i and o mean the inside and outside liposome. The dependency of photoreduction of $\mathrm{Fe(CN)}_6^{3-}$ on the CCCP concentration is interpreted according to this scheme.

Since the proton transport was fast enough at the higher CCCP concentration, the photoreduction rate was determined by the reactions (1) and (2), i.e. k_1k_2/k_{-1}, and the observed rate was independent of the CCCP concentration. On the other hand, in the lower CCCP concentration, the reduction rate was determined by the reactions (3) and (4), i.e. k_3/k_4, and the observed rate depended on CCCP concentration.

The present study demonstrated the importance of ion transport coupled with the photoinduced electron transfer across the membrane.

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