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Photoreduction of 2,6-dichlorophenolindophenol by chloroplasts with exogenous Mn^{2+} as electron donor

Stimulation of aerobic photooxidation reactions in isolated chloroplasts by Mn^{2+} is a well-known phenomenon[1-3]. The photooxidation of Mn^{2+} itself by chloroplasts has been demonstrated[4-6]. The mechanism of aerobic Mn^{2+} photooxidation (apparently a complex reaction with a moderate sensitivity to 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU)) has been suggested to include donation of electrons from Mn^{2+} to Photosystem II[5,6]. HOMANN's[7] fluorescence experiments also support this view. These observations are of special interest because of the role of Mn^{2+} in photosynthetic electron transport, which is currently a subject of intensive study[8-11]. However, the rate of Mn^{2+} photooxidation actually obtained was extremely low (<20 μequiv/h·mg chlorophyll). Very recently ITOH *et al.*[12] have reported that Mn^{2+} stimulated the residual activity of 2,6-dichlorophenolindophenol (DCIP) photoreduction in Tris-washed[13] chloroplasts, but the enhanced rate (about 30 μequiv/h·mg chlorophyll) was again far below normal Hill reaction rates. This communication describes our independent observation that, with EDTA-treated chloroplasts in which the water-oxidation mechanism has been destroyed by heat treatment, a rapid DCIP photoreduction (which rate is comparable to Hill reaction rates) does occur when Mn^{2+} is the sole electron donor.

EDTA-washed, Cl^--depleted chloroplasts were prepared from commercial spinach (*Spinacia oleracea* L.) as described before[14] and heated at 52° for 2 min to destroy normal Hill reaction activity. Or, alternatively, leaves were heated in hot water (52°) for 2 min before grinding and chloroplasts were isolated by the same procedure. In general, the latter method (*cf.* ref. 7) yielded slightly more active chloroplasts. In either method, chloroplasts were finally washed twice with distilled water and stored in distilled water. The repeated washing of chloroplasts was to thoroughly remove EDTA and extractable reducing substances (*e.g.* ascorbic acid) from the chloroplasts. The reduction of DCIP (freshly recrystallized) was assayed by recording the absorbance changes of reaction mixtures at 580 nm. The actinic light used was a rate-saturating red light (620–700 nm). The temperature was 19°.

Chloroplasts prepared as above have no Hill reaction activity as shown by their inability to reduce DCIP in the light (even in the presence of Cl^-). However, if Mn^{2+} (as $MnCl_2$ or $MnSO_4$) is added the reduction of DCIP does occur. The dye reduction, unlike normal Hill reactions, does not proceed to completion presumably because of a non-biological back oxidation of reduced DCIP by the oxidation product (Mn^{3+}?) of Mn^{2+}. The rate of dye reduction estimated from the initial slopes of recorded curves is proportional to the amount of chloroplasts present in the reaction mixture (Fig. 1), varying from 150 to 250 μequiv/h·mg chlorophyll depending on the chloroplast preparation. The higher rates, which are nearly 10 times greater than those obtained by other workers, lie well within the range of normal Hill reaction rates. Fig. 2a shows that the observed absorbance changes indeed represent the reduction of DCIP, thus eliminating the possibility that the changes were due to light-scattering changes of

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCIP, 2,6-dichlorophenolindophenol; HEPES, *N*-hydroxyethylpiperazine-*N'*-ethanesulphonic acid; MES, *N*-morpholinoethanesulphonic acid; HEPPS, *N*-hydroxyethylpiperazine-*N'*-propanesulphonic acid.

chloroplasts. In fact, if the chloroplasts have not been washed with EDTA a Mn^{2+}-dependent light-scattering change (an increase in turbidity) does occur upon illumination, a change large enough to obscure the kinetics of DCIP photoreduction. The scattering change, however, can be suppressed by ammonia (10 mM). The Mn^{2+}-supported DCIP photoreduction has a pH optimum at pH 7.5–8.0 (Fig. 2b) which is close to the pH optimum for the Hill reaction in EDTA-washed chloroplasts[14].

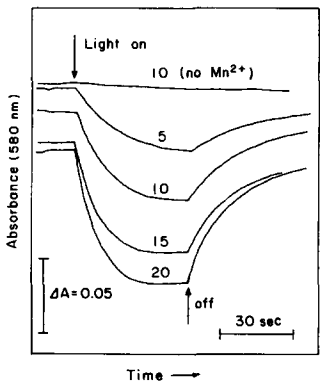

Fig. 1. Kinetics of Mn^{2+}-supported DCIP photoreduction in EDTA-treated, heated chloroplasts. The figures 5–20 represent the amount of chloroplast present in the reaction mixture (μg chlorophyll per ml). The reaction mixture (2 ml) contained *N*-hydroxyethylpiperazine-*N'*-ethanesulphonic acid (HEPES)–NaOH buffer, 40 μmoles (pH 7.5); DCIP, 0.04 μmole; $MnSO_4$, 0.2 μmole. The initial slopes correspond to a DCIP reduction rate of approx. 150 μequiv/h·mg chlorophyll. Actinic light, 620–700 nm.

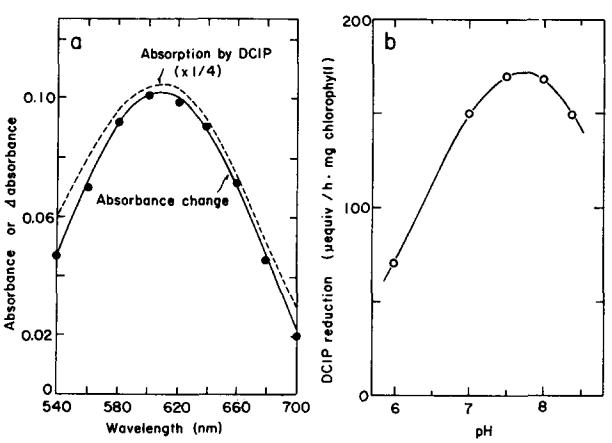

Fig. 2. a. Comparison of difference spectrum (dark *minus* light) with the absorption spectrum of DCIP. In this particular case a blue actinic light (420 ± 20 nm) was used. The reaction mixture contained 15 μg chlorophyll per ml. Other conditions were the same as for Fig. 1. b. Effect of pH on Mn^{2+}-supported DCIP photoreduction. Chlorophyll, 15 μg/ml. The buffers used were: pH 6.0, *N*-morpholinoethanesulphonic acid (MES)–NaOH; pH 7.0–7.5, HEPES–NaOH; pH 8.0–8.3, *N*-hydroxyethylpiperazine-*N'*-propanesulphonic acid (HEPPS; N. E. Good, unpublished)–NaOH. The basic conditions were as in Fig. 1. Chlorophyll, 15 μg/ml.

The reaction is highly sensitive to DCMU (Fig. 3a) but is not accompanied by any detectable O_2 production; therefore the restoration of DCIP reduction by the addition of Mn^{2+} is not due to a reactivation of the Hill reaction. It seems safe to conclude that in these EDTA- and heat-treated chloroplasts exogenous Mn^{2+} can serve as an efficient electron donor for Photosystem II. There was no indication that more reducing Fe^{2+} supports DCIP photoreduction. (Fe^{2+} reduces DCIP chemically at a measurable rate, which, however, was not influenced by the presence of illuminated chloroplasts.)

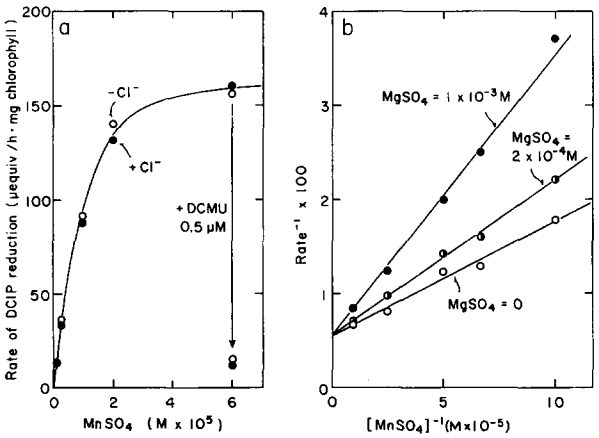

Fig. 3. a. Dependence of DCIP photoreduction upon Mn^{2+} concentration and the lack of Cl^- effect. Cl^- was added as NaCl (5 mM). The reaction conditions were as in Fig. 1. Chlorophyll, 15 μg/ml. b. Competitive inhibition of Mn^{2+}-supported DCIP photoreduction by Mg^{2+}. The basic conditions were as in Fig. 1. Chlorophyll, 15 μg/ml reaction mixture.

The affinity of Mn^{2+} to its reaction site is rather high—the apparent Michaelis constant for Mn^{2+} being about $1.2 \cdot 10^{-5}$ M at pH 7.5 (Figs. 3a and 3b). Mg^{2+} was found to act as a typical competitive inhibitor of this reaction. It can be estimated from Fig. 3b that the reaction site combines with Mg^{2+} with an apparent dissociation constant of approx. $7 \cdot 10^{-5}$ M, an affinity equivalent to about 1/6 of that for Mn^{2+}. EDTA (1 mM) added to the reaction mixture completely inhibits the DCIP photoreduction.

The Mn^{2+}-supported DCIP photoreduction is not stimulated by Cl^- (Fig. 3a). The involvement of Cl^- in the Hill reaction[15] was recently reconfirmed and the Cl^--requiring step was located close to the terminal water oxidation mechanism of Photosystem II[14]. The lack of Cl^- effect on the Mn^{2+} reaction seems to indicate that the site of intervention of exogenous Mn^{2+} is located between the Cl^--requiring step and Photoact II. It seems possible that the component of the heated chloroplasts which reacts with exogenous Mn^{2+} (or combines with Mg^{2+} if forced) represents a denatured 'apoenzyme' of the Mn complex(es) originally involved in the mechanism of water oxidation[9].

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Department of Biology, Queen's University, Kingston, Ontario (Canada) S. IZAWA*

1 A. H. MEHLER, *Arch. Biochem. Biophys.*, 34 (1951) 339.
2 H. M. HABERMANN AND H. GAFFRON, *Photochem. Photobiol.*, 1 (1962) 159.
3 P. H. HOMANN, *Biochemistry*, 4 (1965) 1902.
4 R. H. KENTEN AND P. J. G. MANN, *Biochem. J.*, 61 (1955) 279.
5 J. M. MCKENNA AND N. I. BISHOP, *Biochim. Biophys. Acta*, 131 (1967) 339.
6 R. BACHOFEN, *Brookhaven Symp. Biol.*, 19 (1966) 478.
7 P. H. HOMANN, *Biochem. Biophys. Res. Commun.*, 33 (1968) 229.
8 G. M. CHENIAE AND I. F. MARTIN, *Brookhaven Symp. Biol.*, 19 (1966) 406.
9 B. KOK AND G. M. CHENIAE, *Abstr. 11th Intern. Botany Congr., Seattle, 1969*, p. 114.
10 R. H. LOZIER AND W. L. BUTLER, *Abstr. 11th Intern. Botany Congr., Seattle, 1969*, p. 132.
11 G. HIND, H. M. NAKATANI AND R. L. HEATH, *Abstr. 11th Intern. Botany Congr., Seattle, 1969*, p. 91.
12 M. ITOH, K. YAMASHITA, K. NISHI AND K. SHIBATA, *Biochim. Biophys. Acta*, 180 (1969) 509.
13 T. YAMASHITA AND W. L. BUTLER, *Plant Physiol.*, 43 (1968) 1978.
14 S. IZAWA, R. L. HEATH AND G. HIND, *Biochim. Biophys. Acta*, 180 (1969) 388.

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* Present address: Department of Botany and Plant Pathology, Michigan State University, East Lansing, Mich. 48823, U.S.A.