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Studies on the Component Responsible for the Oxidation of the Primary Electron Acceptor of Photosystem II in the Presence of 3-(3′,4′-Dichlorophenyl)-1,1-Dimethyl Urea: Reactivity to External Reductants

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The dark reoxidation of the photochemically reduced primary electron acceptor of photosystem II, Q, in the presence of 3-(3′,4′-dichlorophenyl)-1,1-dimethyl urea (DCMU) by the redox counterpart (here designated Z) of Q was studied by monitoring the dark recovery of the induction of chlorophyll fluorescence.

In normal chloroplasts, the dark reoxidation of reduced Q in the presence of DCMU was not affected by the externally added hydrophilic reductants; ascorbate, hydroquinone, hydrogen peroxide, manganous chloride, potassium iodide and potassium ferrocyanide. In chloroplasts whose oxidizing side of photosystem II had been inactivated by heat- or Tris-treatments, reoxidation was inhibited partially. This inhibition increased on the addition of hydrophilic reductants, but was relieved by increasing the redox potential of the suspension medium with the chloroplasts.

We concluded that the redox counterpart, Z, of Q in the presence of DCMU is located in a hydrophobic environment which can be denatured by heat- or Tris-treatments to allow the access of normally extruded hydrophilic electron donors.

Key words: Chloroplasts — DCMU — Electron donors — Photosystem II — Primary acceptor (photosystem II) — Reoxidation of Q.

The photochemically reduced primary electron acceptor of photosystem II, Q, normally is oxidized by the plastoquinone pool, A. In the presence of an inhibitor, however, such as DCMU which blocks electron transport between Q and A, reduced Q is reoxidized by an unknown redox component (here designated Z) on the oxidizing side of photosystem II (Bennoun 1970, Homann 1971, Ikegami and Katoh 1973). This reoxidation of reduced Q, monitored by the dark recovery of the fluorescence induction of chlorophyll, was inhibited when the oxidizing side of photosystem II was blocked by hydroxylamine (Bennoun 1970, Homann 1971), CCCP (Homann 1971, Ikegami and Katoh 1973), or Tris-treatment (Homann 1971).

In contrast, the effect of artificial electron donors for photosystem II on the

Abbreviation: CCCP, carbonylcyanide *m*-chlorophenylhydrazone.

reoxidation of Q in the presence of DCMU is ambiguous. Homann (1971) showed that dark restoration of fluorescence induction in normal chloroplasts was inhibited by the artificial electron donors for photosystem II, hydrogen peroxide, ascorbate-*p*-phenylenediamine couple and ascorbate-N,N,N′,N′-tetramethyl-*p*-phenylenediamine couple, but not by manganous sulfate. He also stated that manganous ions to some extent affected dark restoration in Tris-treated chloroplasts. Ikegami and Katoh (1973) reported that the ascorbate-N,N,N′,N′-tetramethyl-*p*-phenylenediamine couple was effective in suppressing dark restoration in normal chloroplasts, but the ascorbate-*p*-phenylenediamine couple and manganous sulfate were ineffective.

We here show that hydrophilic electron donors, including ascorbate and the manganous ion, inhibited reoxidation of reduced Q only when the thylakoid membrane had been damaged by heat- or Tris-treatment, and that the reoxidation of Q in heated chloroplasts depends on the redox potential of the medium surrounding the thylakoid membrane.

Materials and Methods

Spinach chloroplasts were prepared as described previously (Okayama 1976). Heated chloroplasts were prepared by incubation of chloroplasts equivalent to 2 mg chlorophyll that were suspended in 1 ml of 50 mM Tris buffer (pH 7.5) containing 10 mM NaCl and 400 mM sucrose at 50°C for 4 min in the dark. Tris-treatment of chloroplasts was done by the standard method (Yamashita and Butler 1968).

For the determination of fluorescence intensity, chloroplast preparations (2 mg chlorophyll/ml of buffer) were diluted with 50 mM Tricine buffer (pH 7.2) containing 20 mM KCl, 5 mM $MgCl_2$ and 10 μM DCMU to an equivalence of 10 μg chlorophyll per ml. The induction of fluorescence upon admission of an excitation light by a mechanical shutter (opening time approximately 2 msec) first was recorded on a transient recorder (TCA 2000, Riken Denshi Co., Ltd.) then on a stripchart recorder. The fluorescence excitation light (about 5×10^4 ergs/cm^2·sec) was obtained from a d. c. operated xenon lamp combined with a blue filter (Hoya B440) and a 3 cm layer of 10% $CuSO_4$ solution. The fluorescence emission at 682 nm, isolated by an interference filter with a half band width of 15 nm, was detected with S-20 type phototube.

Redox potentials of chloroplast suspensions in 50 mM Tricine buffer (pH 7.2) containing 20 mM KCl, 5 mM $MgCl_2$, 10 μM DCMU and 0.1 mM potassium ferrocyanide were measured with a combination Pt/Ag-$AgCl_2$ electrode (Horiba, No. 6810-05T), then adjusted by addition of 1–10 μl portions of the oxidant (0.5 M potassium ferricyanide), as described previously (Okayama and Butler 1972). All titrations were performed aerobically at 25°C in the dark.

Results

The fluorescence yield of chlorophyll *a* in dark-adapted chloroplasts increased from the initial level, F_o, to the steady state level, F_s, during illumination for 1–2 sec with a strong blue light. The area above this fluorescence-rise curve in the presence

of DCMU is known to be proportional to the amount of Q (Murata et al. 1966). In this study, the ratio of reduced Q, Q^-, to the total amount of Q, Q_t, was estimated from the initial fluorescence level, F_i, of the chloroplasts at a given point in the dark period after a previous illumination for 5 sec, using the following equation (Malkin and Kok 1966, Itoh 1978).

$$[Q^-]/[Q]_t=(F_s-F_i)/(F_s-F_o)$$

Fig. 1 shows that blockage of the oxidizing side of photosystem II by CCCP or hydroxylamine caused the inhibition of dark reoxidation of photoreduced Q in the presence of DCMU. Ascorbate did not affect reoxidation regardless of the presence or absence of CCCP. Essentially the same results have been reported by others (Bennoun 1970, Homann 1971, Ikegami and Katoh 1973). Mild treatment of chloroplasts with heat has been reported to block the oxidizing side of photosystem II as Tris-treatment did (Katoh and San Pietro 1967, Babcock and Sauer 1975). The dark reoxidation of reduced Q in the presence of DCMU in heated chloroplasts was partially inhibited, and this inhibition was increased by ascorbate, manganous chloride, or potassium ferrocyanide, as seen in Fig. 2a. Other artificial electron donors for photosystem II, hydroquinone, potassium iodide and hydrogen peroxide, also increased inhibition of the dark reoxidation in heated chloroplasts (Fig. 2b). Fig. 3 shows that in normal chloroplasts none of the electron donors used affected the dark reoxidation of reduced Q in the presence of DCMU. Inhibition of the

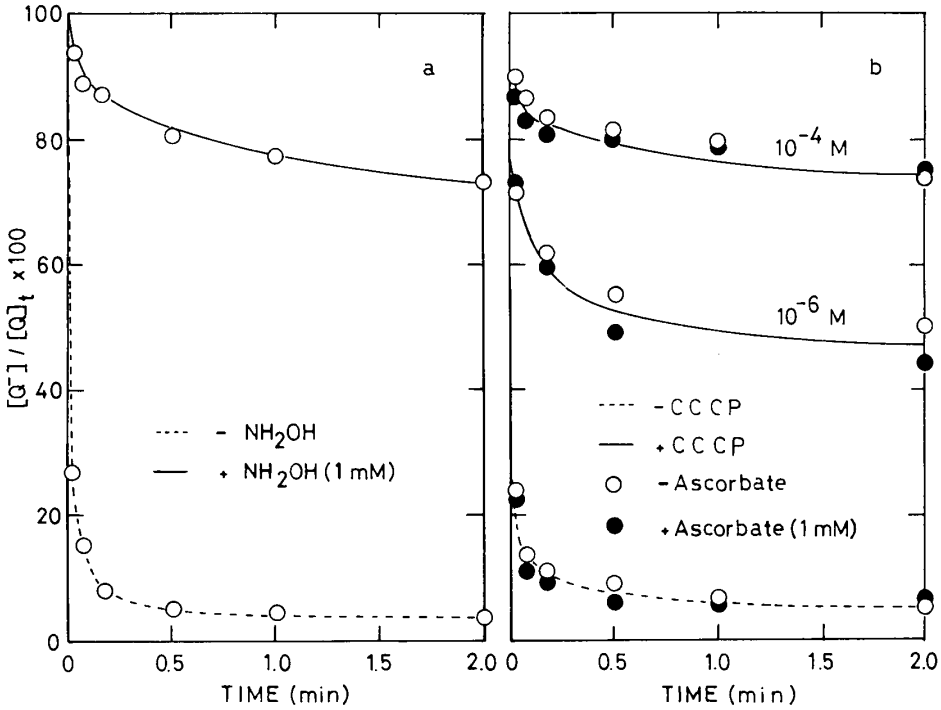

Fig. 1 Inhibition of the reoxidation of reduced Q in the presence of DCMU by hydroxylamine and by CCCP. Broken lines, no addition. Solid lines, in the presence of 1 mM NH_2OH (a), and 10^{-4} M or 10^{-6} M CCCP (b). Closed circles, in the presence of 1 mM ascorbate. Open circles, without ascorbate. The dark reoxidation of Q^- was determined at a given point in the dark period (abscissa) after a previous illumination.

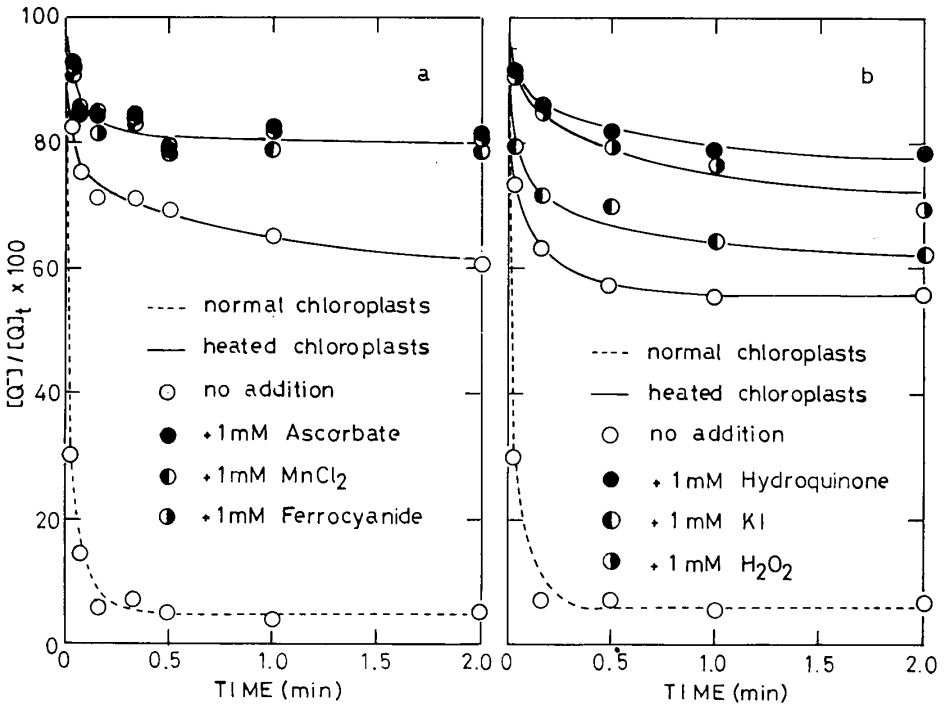

Fig. 2 Inhibition of the reoxidation of reduced Q in the presence of DCMU by heat-treatment. Broken lines, untreated chloroplasts. Solid lines, chloroplasts heated for 4 min at 50°C. Open circles, no addition. Closed or semiclosed circles, 1 mM ascorbate, manganous chloride, potassium ferrocyanide (a), hydroquinone, potassium iodide, and hydrogen peroxide (b), as indicated.

dark reoxidation also took place with Tris-treated chloroplasts, and inhibition was increased further by ascorbate (data not shown). These results indicate that the hydrophilic electron donors for photosystem II affect the reoxidation of reduced Q only when chloroplasts have been treated with heat or Tris.

In heated chloroplasts the reoxidation of reduced Q in the presence of DCMU depended on the redox potentials of the chloroplast suspensions (Fig. 4). At the lower potential (250 mV), reoxidation was almost completely inhibited. As the potentials increased (above 450 mV), inhibition of the dark reoxidation of reduced Q by heat-treatment was relieved. These results suggest that the Z in heated chloroplast is mostly in the reduced form, which results in inhibition of the reoxidation of reduced Q in the presence of DCMU.

Discussion

In the presence of DCMU, photoreduced Q is oxidized by a component on the oxidizing side of photosystem II (Bennoun 1970, Homann 1971, Ikegami and Katoh 1973). Thus, the component designated Z in this paper, could be an electron carrier between photosystem II and water, and might be identical to the Signal II_f component which produced a rapid light-induced reversible electron spin resonance signal (Babcock and Sauer 1975). In the presence of DCMU, Signal II_f in previously illuminated Tris- or heat-treated chloroplast suspensions of low redox poise (less than 400 mV) was inhibited, but at higher potentials inhibition was relieved

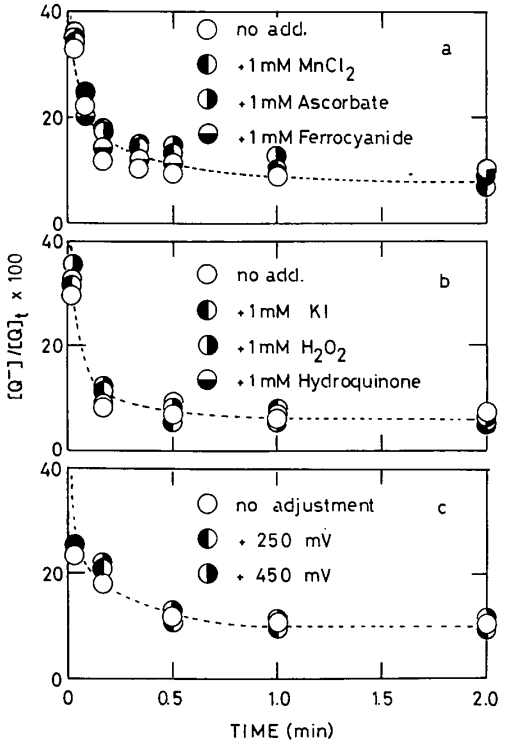

Fig. 3

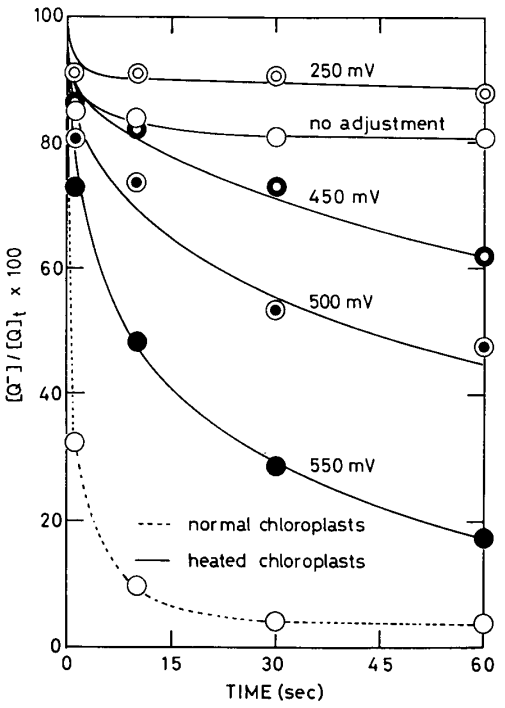

Fig. 4

Fig. 3 Effects of hydrophilic reductants (a, b) and redox potentials (c) on the dark oxidation of reduced Q in untreated chloroplasts in the presence of DCMU. At time zero, $[Q^-]/[Q]_t=1$. Open circles, no addition. Closed or semiclosed circles, in the presence of 1 mM manganous chloride, ascorbate, potassium ferrocyanide, potassium iodide, hydrogen peroxide and hydroquinone as indicated (a, b), and at the redox potentials poised by ferricyanide and ferrocyanide at +250 and +450 mV.

Fig. 4 Effects of redox potentials on the dark oxidation of reduced Q in heated chloroplasts in the presence of DCMU. Broken line, untreated chloroplasts without adjustment of redox potentials. Solid lines, heated chloroplasts poised at the potentials indicated by potassium ferricyanide and ferrocyanide.

(Babcock and Sauer 1975). Inhibition of the dark reoxidation of reduced Q in the presence of DCMU in heated chloroplasts also was relieved at redox potentials higher than 450 mV (Fig. 4). These results suggest that in the presence of DCMU reduced Q is oxidized by the Signal II_f component, Z^+, at higher potentials; whereas, at lower potentials Z stays in the reduced form, resulting in the inhibition of the oxidation of reduced Q.

Redox potentials shown in Fig. 4 were poised by potassium ferricyanide and ferrocyanide without a lipophilic redox mediator. As seen in Fig. 1–3, other hydrophilic reductants; ascorbate, hydroquinone, hydrogen peroxide, potassium iodide and manganous chloride increased the inhibition of the reoxidation of reduced Q in heated chloroplasts, but they did not affect reoxidation in normal chloroplasts. Possibly, the Z in normal chloroplasts is located in a hydrophobic environment to which hydrophilic reductants can not gain access. The hydrophobic area might be denatured by heat- or by the Tris-treatment of chloroplasts to allow the access of hydrophilic reagents to Z.

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