Inhibition of Oxygen Evolution in Chloroplasts by Ferricyanide

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ABSTRACT
Preincubation of chloroplasts from pea leaves (Pisum sativum L. cv. Kelvedon) with 0.5 millimolar ferricyanide in the dark, caused a parallel inhibition of the rate of rise of the variable fluorescence and the rate of electron transport. Both reactions were inhibited to a similar extent by varying the time of preincubation, the concentration of ferricyanide during preincubation, and by raising the concentration of salts in the preincubation medium. Ferricyanide treatment of Tris-washed chloroplasts did not inhibit electron transport from the Photosystem II (PSII) electron donor 1,5-diphenylcarbazide to methylviologen. The inhibition of the variable fluorescence rise and of NADP reduction (caused by ferricyanide pretreatment) was bypassed by addition of the PSII electron donor couple hydroquinone/methylviologen. It was concluded that preincubation of chloroplasts with ferricyanide in the dark inhibited electron transport between water and PSII.

Brewer and Jagendorf (3) observed that dark preincubation of chloroplasts with 0.5 mM FeCN caused an inhibition of electron transport which they attributed to a step related to O₂ evolution. Ikegami and Katoh (10) have found that preincubation of chloroplasts with FeCN slowed down the rise kinetics of fluorescence in the presence of DCMU. This was interpreted as due to oxidation of an internal acceptor during preincubation. Contrary to the interpretation of Brewer and Jagendorf (3), they suggested that the site inhibited is located between the reaction center of PSII and the DCMU-sensitive site.

In the present work, the effects of preincubation with FeCN on electron transport and fluorescence were reinvestigated and compared. We conclude that the interpretation of Brewer and Jagendorf (3) is valid and that the inhibition in the rate of the fluorescence rise is also caused by inhibiting electron flow between H₂O and PSII.

MATERIALS AND METHODS
Isolation of Chloroplasts. Chloroplasts were prepared from 2-week-old pea seedlings (Pisum sativum L. cv. Kelvedon) by a modified method of Nakatani and Barber (11). Ten g of leaves were homogenized for 5 s in a homogenizer with 75 ml of medium containing 0.33 M sorbitol, 0.2 mM MgCl₂, and 20 mM Mes, pH 6.5. The homogenate was filtered through 8 layers of gauze and centrifuged at 2500 g for 40 s. The pellet was resuspended in the same medium.

Tris Treatment. The chloroplast pellet was resuspended in 16 ml of 0.8 M Tris, pH 8, and incubated on ice for 25 min. Thereafter, the suspension was centrifuged at 10,000 g for 7 min and the pellet was resuspended in the sorbitol-MgCl₂-Mes medium.

Chl was determined following the method of Arnon (1).

Preincubation Media. Aliquots of chloroplasts containing 13 to 100 μg Chl were resuspended in 1 or 1.5 ml of distilled H₂O to which was added, immediately, an equal volume of a twice concentrated reaction mixture giving a final concentration of 100 mM sorbitol, 300 mM KCl, 5 mM Heps, pH 7.9. The chloroplast suspension was subsequently preincubated with or without FeCN, as indicated.

Assays. Electron transport with FeCN or MV was measured by monitoring the changes in O₂ concentration with a YSI Clark type O₂ electrode at 25°C. The samples were illuminated with a 500-w projector lamp providing white light at an intensity of 1.9 × 10⁴ ergs cm⁻² s⁻¹ at the center of the vessel.

For NADP reduction, illumination was provided with red light at an intensity of 4.5 × 10⁴ ergs cm⁻² s⁻¹ and electron flow was measured by following the absorbance changes at 350 nm.

Fluorescence induction measurements were made with a specially constructed instrument similar to that described by Epel and Levine (7). Excitation was performed by a light beam at right angles to the photodetector (EMI 9558 S-20 sensitivity). The exciting beam passed through a 630 nm interference filter (Schott; half band, 14 nm) providing a light intensity of 250 ergs cm⁻² s⁻¹. The fluorescence emission was isolated by use of blocking filters (RG 715, RG N-9, RG 665) transmitting above 710 nm with 50% transmission at 730 nm. The signal was recorded on a Rickadenki fast response recorder.

RESULTS
Figure 1 shows the fluorescence rise upon illumination of chloroplasts pretreated with FeCN and for comparison, chloroplasts pretreated in the same medium but without FeCN. DCMU, 10 μM, was added 15 s prior to the onset of illumination. Pretreatment with FeCN inhibited markedly the rate of the rise of the Fv. The half rise time (t₁/₂) of the Fv was 1.65 s in FeCN pretreated chloroplasts as compared to 1.0 s in chloroplasts pretreated without FeCN. The extents of the Fo and Fv were unaffected. These results resemble those reported by Ikegami and Katoh (10).

The effect of preincubation with FeCN on electron flow at various light intensities is shown in Figure 2. Inhibition was obtained at all light intensities, and the degree of inhibition was similar in saturating and limiting light. The effects of various conditions on electron transport and fluorescence kinetics in terms of t₁/₂ were compared. Electron transport and the kinetics of fluorescence rise were inhibited in a parallel manner as a function of ferricyanide concentration during preincubation (Fig. 3).

Both the rate of electron transport and the rate of fluorescence rise were inhibited progressively (in parallel) with time of FeCN preincubation (Fig. 4). Preincubation in the absence of FeCN had little effect on both reactions.

Addition of KCl to the preincubation medium enhanced the inhibitory effect of FeCN (Fig. 5). Again, both reactions were affected in a similar way.

The extent of inhibition of electron flow (due to preincubation

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1 Abbreviations: FeCN, ferricyanide; Fo, invariable fluorescence; Fv, variable fluorescence; DPC, 1,5-diphenylcarbazide, MV, methylviologen.
INHIBITION OF O₂ EVOLUTION IN CHLOROPLASTS BY FERRICYANIDE

FIG. 1. The effect of FeCN on fluorescence. The preincubation medium contained in a volume of 3 ml: 5 mM HEPES, pH 7.9; 100 mM sorbitol; 300 mM KCl; and chloroplasts equivalent to 13 μg Chl. FeCN, when added to the preincubation medium, 0.5 mM. Preincubation time, 5 min. DCMU (10 μM) was added 15 s prior to the onset of illumination.

FIG. 2. The effect of FeCN on electron transport. The preincubation medium contained in a volume of 2 ml: 5 mM HEPES, pH 7.9; 100 mM sorbitol; 300 mM KCl; chloroplasts equivalent to 50 μg Chl. FeCN, when added to the preincubation medium, 0.5 mM. Preincubation time, 5 min. NH₄Cl (2.5 mM) was added 20 s prior to the onset of illumination. FeCN (0.5 mM) was added to samples preincubated without it 10 s prior to the onset of illumination. Maximal light intensity, 1.9 × 10⁵ ergs cm⁻² s⁻¹.

FIG. 3. The effect of various FeCN concentrations in preincubation medium on fluorescence and electron transport. Preincubation time, 4 min. In the electron transport experiments, FeCN was added up to 0.5 mM (both in the experiments pretreated with and without FeCN) at the end of the preincubation. The reaction was measured at light intensity of 1.9 × 10⁵ ergs cm⁻² s⁻¹. Other details as in Figures 1 and 2. Control values: 114 μmol O₂/mg Chl·h; t₁/₂, half rise time of Fv, 1.25 s.

corresponding to a potential of 350 mV. Similar values were obtained for the inhibition of electron flow by Brewer and Jagen-dorf (3) and by Ikegami and Kato (8) for the fluorescence change (10). The fact that the extent of inhibition increased with rise of redox potential indicates that the inhibition is due to an oxidation of a factor involved in electron transport.

In order to localize the possible site of inhibition, we investigated the effect of FeCN pretreatment on electron flow from DPC (13) to MV in Tris-treated chloroplasts (14). However, since DPC reduces FeCN chemically, such a system can be used only if the FeCN inhibition can be maintained even after FeCN was removed from the chloroplasts. FeCN was removed by sieving the chloroplasts on a Sephadex G-50 column; (sieving removed approximately 95% of the FeCN). As can be seen from the results of

with FeCN) in the presence of salts was dependent on the type of anion present (Fig. 6). The degree of inhibition by FeCN increased in the order I⁻ > NO₃⁻ > Cl⁻. Fifty % inhibition was caused by 30 mM KI, 60 mM KNO₃, and 140 mM KCl. No significant effects were caused by changing the type of cation (not shown).

The extent of inhibition increased with increase in the ferri- to ferrocyanide ratio in the preincubation medium. An inhibition of 50% of electron flow was obtained at a ferri- to ferrocyanide ratio

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This page contains a scientific diagram and text discussing the effect of preincubation time on electron transport and fluorescence in chloroplasts. The text explains that the rate of electron transport can be measured at a light intensity of 1.9 × 10^6 ergs cm⁻² s⁻¹. The rate of NADP oxidation by DPC is shown to be influenced by salt concentration, with figures illustrating the effect of different anions on preincubation with FeCN.

Table I describes the effect of FeCN on electron transport in control and Tris-treated chloroplasts. Chloroplasts equivalent to 200 μg Chl were preincubated in 4 ml of medium, as described in "Materials and Methods," for 6 min with or without 1.0 mM FeCN. The preincubation chloroplast suspension was loaded on a Sephadex G-50 coarse column (1.6 cm diameter, 6 cm length) equilibrated with 100 ml of the same medium. The chloroplasts were eluted with the preincubation medium. 1.8 ml from the first 2 ml eluate was transferred into the electrode vessel. The reaction mixture included in a final volume of 1.9 ml: 0.2 mM MV, 0.5 mM NaN₃, and 2.5 mM NH₄Cl. DPC at 0.5 mM was added to the Tris-treated chloroplasts as a donor. Chl concentration was determined in each sample eluted.

The effect of FeCN on electron transport and fluorescence is shown in Figure 4, which illustrates the percentage of electron transport at various KCl concentrations. Figure 5 shows the effect of preincubation with FeCN at various KCl concentrations on electron transport and fluorescence. The electron transport rate was measured at a light intensity of 1.9 × 10^6 ergs cm⁻² s⁻¹. The values of t₁/₂ and electron transport of the samples which were preincubated without FeCN were taken as 100% at each salt concentration. Other details as in Figures 1 and 2.

Ascorbate couple is shown for the fluorescence kinetics. Preincubation with FeCN increased t₁/₂ (i.e., slowed the rise kinetics) of the Fv from 1.3 to 2.3 s. Addition of hydroquinone and ascorbate decreased t₁/₂ to 1.7 s (Table III). Ascorbate alone had no effect; thus, the effect cannot be due to a chemical reduction of ferricyanide by ascorbate.

**DISCUSSION**

It has been reported previously that preincubation of chloroplasts with FeCN causes a decrease in the rate of rise of the Fv. This effect was explained as being due to oxidation of an acceptor between the reaction centre of PSII and the DCMU-sensitive site (10). The acceptor, when oxidized by FeCN, could presumably oxidize Q, the primary acceptor of PSII, and inhibit the rise of the Fv. The same explanation was given by Bowes et al. (2), though
an inhibition at the side of the donor of PSII has not been excluded. However, we found that the changes in fluorescence and electron transport were parallel when measured under the same conditions of preincubation (Figs. 3–5) suggesting the involvement of the FeCN-sensitive component in both reactions. Thus, the simplest explanation for the inhibition in the fluorescence rise (increase in $I_{1/2}$) would be that it is caused by inhibition of electron flow from H$_2$O to PSII. The analysis and explanation of Ikegami and Katoh (10) is based on the prevalent assumption that the rate of electron flow from H$_2$O to PSII is not limiting.

Our explanation is supported by the results presented in Tables I to III. Electron flow from DPC to MV in Tris-treated chloroplasts is not affected by FeCN treatment. Moreover, both the rate of electron flow and the rate of fluorescence rise were markedly increased by restoring electron flow with the hydroquinone ascorbate couple.

Ikegami and Katoh (10) found that Tris-treated chloroplasts were inhibited by the FeCN treatment, a result which differs from our findings. Since, in their experiment, apparently no alternative donor to PSII was added, we suggest that the fluorescence measured in their experiment was due to an incomplete inhibition of electron donation by water.

In our study, and from previous work (3, 10), it seems that the factor oxidized by FeCN (involved in inhibition) has a redox potential in the vicinity of 350 mv. A known carrier present in the chloroplast and having a similar redox potential is Cyt b$_{559}$ HP (6). Its involvement in the inhibition may be postulated on the basis of its proximity to the water oxidizing system, its high redox potential, and the lability of its potential to treatments that inhibit water donation (5, 6). However, the location of Cyt b$_{559}$ HP in the membrane seems to be near the surface (9) and no lag was seen when oxidized by FeCN (10). Thus, if this factor is oxidized by FeCN preincubation, it presumably leads to other changes which are the direct cause for inhibition of electron flow and decrease in the rate of fluorescence rise. In addition, it should be mentioned that the possible participation of Cyt b$_{559}$ HP in electron transport is controversial (6).

The fact that inhibition by FeCN is not reversed by ascorbate, its slow development, and its persistence after removal of FeCN indicates that the damage caused is irreversible and that oxidation by FeCN is probably only the initial step which leads to inhibition of electron flow and inhibition of the rise kinetics of fluorescence.

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