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# EFFECTS OF CHLORIDE DEPLETION ON ELECTRON DONATION FROM THE WATER-OXIDIZING COMPLEX TO THE PHOTOSYSTEM II REACTION CENTER AS MEASURED BY THE MICROSECOND RISE OF CHLOROPHYLL FLUORESCENCE IN ISOLATED PEA CHLOROPLASTS

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**The role of $Cl^-$ in the electron transfer reactions of the oxidizing side of Photosystem II (PS II) has been studied by measuring the fluorescence yield changes corresponding to the reduction of $P^+$-680, the PS II reaction center chlorophyll, by the secondary PS II donor, Z. In $Cl^-$-depleted chloroplasts, a rapid rise in fluorescence yield was observed following the first and second flashes, but not during the third or subsequent flashes. These results indicate that there exists an additional endogenous electron donor beyond P-680 and Z in $Cl^-$-depleted systems. In contrast, the terminal endogenous donor on the oxidizing side of PS II in Tris-washed preparations has previously been shown to be Z, the component giving rise to EPR signals $II_f$ and $II_{vf}$. The rate of reduction of $P^+$-680 in the $Cl^-$-depleted chloroplasts was as rapid as that measured in uninhibited systems, within the time resolution of our instrument. Again, this is in contrast to Tris-washed preparations in which a dramatic decrease in the rate if this reaction has been previously reported. We have also carried out a preliminary study on the rate of rereduction of $Z^+$ in the $Cl^-$-depleted system. Under steady-state conditions, the reduction half-time of $Z^+$ in uninhibited systems was about 450 $\mu$s, while in the $Cl^-$-depleted chloroplasts, the reduction of $Z^+$ was biphasic, one phase with a half-time of about 120 ms, and a slower phase with a half-time of several seconds. The appearance of the quenching state due to $P^+$-680 observed following the third flash on excitation of $Cl^-$-depleted chloroplasts was delayed by two flashed when low concentrations of $NH_2OH$ (20–50 $\mu$M) were included in the medium. Hydrazine at somewhat higher concentrations showed the same effect. This is taken to indicate that the reactions leading to PS II oxidation of $NH_2OH$ or $NH_2NH_2$ are uninhibited by $Cl^-$ depletion. Addition of $NH_2OH$ at low concentrations to Tris-washed chloroplasts did not alter the pattern of the fluorescence yield, indicating that the reactions leading to the $NH_2OH$ oxidation present in $Cl^-$-depleted systems are absent following Tris inhibition. The results are discussed in terms of an inhibition by $Cl^-$ depletion of the reactions of the oxygen-evolving complex. It is suggested that no intermediary redox couple exists between the oxygen-evolving complex and Z, and that $Z^+$ is reduced directly by Mn of the complex. In terms of the S-state model, $Cl^-$ depletion appears to inhibit the advancement of the mechanism beyond $S_2$, but not to inhibit the transitions from $S_0$ to $S_1$, or from $S_1$ to $S_2$.**

## Introduction

It has long been known that $Cl^-$ stimulates the Hill reaction in isolated chloroplasts [1]. Izawa et al. [2] demonstrated that washing chloroplasts with

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Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; PS II, Photosystem II.

a $Cl^-$-free, low-salt medium (+EDTA at pH 7.2) almost completely inhibited electron donation from water to PS II. This inhibition could be removed by readdition of $Cl^-$, with an apparent activation constant of 0.9 mM chloride at pH 7.2 [3]. $Cl^-$ depletion did not inhibit electron donation to PS II by artificial electron donors [2,3]. These results suggested that the site of activation by $Cl^-$ is between the reaction center chlorophyll, P-680, and the water-oxidizing enzyme system.

Recent optical studies of P-680 [4,5] and kinetic EPR studies of PS II [6,7] indicate the existence of at least one electron transport component, Z, between P-680 and the enzyme which accumulates oxidizing equivalents for the oxidation of water (the oxygen-evolving complex, or the S-state system). The component Z is able to reduce $P^+$-680 rapidly, in the submicrosecond range, and to be reduced by each of the S-states with half-times of 400 $\mu$s (by the $S_2$ state), 1 ms (by the $S_3$ state) or less than 100 $\mu$s (by the $S_0$ and $S_1$ states) [6]. Recent EPR studies of the reduction kinetics of $Z^+$ in $Cl^-$-depleted preparations have shown that $Cl^-$ depletion does not change the microwave power saturation properties of $Z^+$, but that the rate of reduction of the free radical is altered [8].

In the present study, $P^+$-680 reduction kinetics were measured by observing the microsecond rise kinetics of the change in chlorophyll fluorescence yield associated with the loss of $P^+$-680 as quencher [9], using the method developed by Duysens et al. [10,11]. By studying the flash number dependence of $P^+$-680 re-reduction, we have investigated the mechanism of inhibition of the oxygen-evolving complex by $Cl^-$ depletion. In normal chloroplasts, the reaction center of PS II changes its redox state through the following reactions, which occur over the microsecond range (Scheme Ia; asterisk denotes photochemical reaction):

Here, $Q_A$ is the primary stable electron acceptor of PS II and also works as a quencher of fluorescence in its oxidized state [12], and $Q_B$ is the secondary acceptor. The fluorescence yield change in the time range less than 10 $\mu$s reflects the rapid reduction of $P^+$-680 by Z. If Z is oxidized before the flash, the reactions shown below are expected (Scheme Ib):

$P^+$-680 formed by light cannot be reduced by $Z^+$, and would be more slowly re-reduced by the back reaction with $Q_A^-$, or through an inhibited forward electron transfer, and no rapid rise in fluorescence yield would be expected. Thus, by measuring the microsecond fluorescence yield rise following flash excitation, the redox state of Z before the flash can be studied, and if successive flashes are given to dark-adapted chloroplasts, the number of turnovers of Z can also be assayed. In chloroplasts in which electron transport between the oxygen-evolving system and P-680 is inhibited, re-reduction of Z will be stopped after a few flashes, after which no rapid fluorescence yield change should be observed. In the present study, it is shown that in chloroplasts incubated in $Cl^-$-free medium, one reducing equivalent is available in addition to Z and P-680. $Cl^-$ depletion has thus been shown to differ from other types of inhibitory treatments such as Tris-washing [13] or hydroxylamine treatment which effectively disconnect Z from its endogenous donors.

## Materials and Methods

*Materials.* Chloroplasts were isolated from leaves of 3–4-week-old pea plants. Leaves were homogenized in a medium comprising 0.4 M sucrose/0.02 M Hepes (pH 7.8)/0.015 M NaCl. The homogenate was filtered through 16 layers of cheesecloth and centrifuged for 10 min at 2000 × *g*.

$$S_n Z\ \text{P-680}\ Q_A Q_B \xrightarrow{*} S_n Z\ P^+\text{-680}\ Q_A^- Q_B \rightarrow S_n Z^+\ \text{P-680}\ Q_A^- Q_B \rightarrow S_{n+1} Z\ \text{P-680}\ Q_A Q_B^- \qquad \text{(Scheme Ia)}$$

(low) (low) (high) (low)

(fluorescence yield)

$$Z^+\ \text{P-680}\ Q_A Q_B \rightarrow Z^+\ P^+\text{-680}\ Q_A^- Q_B \rightarrow \begin{cases} Z^+\ \text{P-680}\ Q_A Q_B\ \text{(back reaction)} \\ \text{or} \\ Z^+\ P^+\text{-680}\ Q_a Q_B^- \rightarrow Z^+\ \text{P-680}\ Q_A Q_B^- \end{cases} \qquad \text{(Scheme Ib)}$$

(low) (low) (low) D $D^+$

The pellets obtained were resuspended in a small volume of medium comprising 0.1 M $Na_2SO_4$/0.05 M Hepes (pH 7.8) ($Cl^-$-free medium) and stored in the dark and on ice until use. Chlorophyll concentrations were measured as in [14].

For all fluorescence experiments, the concentration of chloroplasts was adjusted to 2.5 $\mu$M chlorophyll. In every experiment, stock solutions of chloroplasts were diluted by the reaction medium by a factor of 1000. Thus, the concentration of $Cl^-$ in the '$Cl^-$-free' medium, can be assumed to have been less than 20 $\mu$M.

*Apparatus.* Microsecond fluorescence yield changes were measured by the method described by Bowes et al. [15], using a home-built rapid fluorescence photometer. Fluorescence was excited by blue light from a xenon flash lamp (EG&G, FX-132, duration of 2.5 $\mu$s at half-maximal intensity), screened by a Corning 4-96 filter. Fluorescence was detected by a photodiode (EG&G SGD 444) through a 690-nm interference filter (Corion, 10 nm half-band width). The signal from the photodiode was fed through a fast amplifier circuit [16] into a transient recorder (Biomation 6000, 5 ns per step, 6 bit resolution with an input impedence of 50 ohms), and then transferred to a PDP 11-10 minicomputer. The signal from the dark-adapted chloroplasts was divided by the reference fluorescence signal from chloroplasts which had been preilluminated by several flashes in the presence of DCMU (10 $\mu$M) and $NH_2OH$ (10 mM) (see below). For every set of measurements, the chloroplasts were dark-adapted for more than 10 min. The reaction mixture was changed before each series of flashes, by using a flow-cell system previously described [17]. Four sets of traces obtained from samples of dark-adapted chloroplasts were averaged to obtain one set of traces of microsecond fluorescence rise.

The fluorescence yield at 70 $\mu$s or later times following flash excitation was measured by the double flash fluorescence measuring system described elsewhere [17]. The instrument was equipped with an additional actinic flash lamp which allowed an extra saturating flash to be fired a short time before any one of the main excitation flashes. This made it possible to estimate the rate of the rapid dark reduction of Z, since the fraction of Z reduced before the flash could be measured from the extent of the fluorescence yield increase induced by the flash.

*Measurement of the kinetics of $P^+$-680 reduction.* Four factors are known to quench the fluorescence of chlorophyll *a* in isolated chloroplasts [9–12,18]. These are the oxidized primary stable acceptor ($Q_A$), the oxidized primary donor ($P^+$-680), the reduced intermediate acceptor pheophytin and a light-induced carotenoid triplet ($C^T$). Among these, pheophytin does not seem to contribute to changes in the fluorescence yield in the microsecond range, unless the reaction medium is maintained at a very low redox potential [18]. In the present study, the profile of fluorescence during flash excitation of the chloroplasts preilluminated in the presence of 10 $\mu$M DCMU and 10 mM $NH_2OH$ (conditions under which the reoxidation of $Q_A^-$ is known to be very slow following a flash), was used as a reference (Fig. 1A). Under these conditions, the PS II reaction centers can be assumed to be in the state P-680 $Q_A^-$ in the dark, and no light-induced fluorescence yield change is expected, except that due to quenching by the carotenoid triplet. Thus, the fluorescence profile during the flash for this state can be used to correct for fluorescence quenching by $C^T$. On

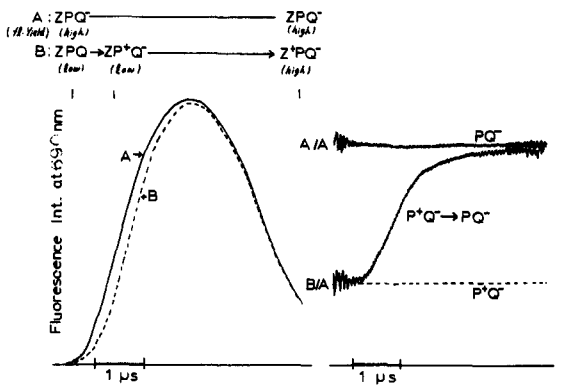



Fig. 1. A schematic representation of the measurement of the microsecond fluorescence yield change. (A) The change in chlorophyll *a* fluorescence intensity during a flash in chloroplasts which have been preilluminated in the presence of 10 $\mu$M DCMU and 10 mM $NH_2OH$. (B) The change in fluorescence intensity in dark-adapted chloroplasts during flash illumination. The trace marked B/A shows the fluorescence yield during a flash, after correction for the quenching by a carotenoid triplet state. Z, P and Q represent the secondary PS II donor, the reaction center chlorophyll and the primary PS II stable acceptor, respectively. For more details, see text.

the other hand, in uninhibited chloroplasts, PS II reaction centers will be in the state P-680 $Q_A$ before a flash in dark-adapted systems (Fig. 1B). If the excitation light intensity is strong enough to turn over all of the reaction centers, the fluorescence yield in the microsecond range will be expected to change due to formation and disappearance of $P^+$-680 and $C^T$. Oxidation of $Q_A^-$ by the secondary acceptor, $Q_B$, is known to proceed with a half-time of 100–200 $\mu$s [17,19], and will not contribute significantly over this time range. Thus, by dividing the fluorescence profile measured during flash excitation of dark-adapted chloroplasts by that of the reference sample (preilluminated in the presence of DCMU and $NH_2OH$), the change in fluorescence yield corresponding to the change of state of the reaction center from $P^+$-680 $Q_A^-$ to P-680 $Q_A^-$ (reduction of $P^+$-680 by the secondary electron donor, Z) can be determined, so long as the formation of $C^T$ under these two conditions is similar. The increase in fluorescence yield in the microsecond range can therefore be interpreted as reflecting the reduction of $P^+$-680, modified by the differential kinetics of formation and decay of $C^T$. Since the formation of $C^T$ depends linearly on the light absorbed in excess of that used for photochemistry [11], its contribution to the kinetics will be minimal at low light intensities. However, the maximal rate of appearance of the high fluorescence state P-680 $Q_A^-$ will depend on a convolution of the rate of photochemical activation, and the rate of reduction of $P^+$-680. A flash intensity was selected which minimized the quenching by $C^T$, but still allowed formation of P-680 $Q_A^-$ in the submicrosecond range.

## Results and Discussion

### *The effect of $Cl^-$ depletion on turnover of the donor side*

Fig. 2 shows the fluorescence yield change in uninhibited (A) and in $Cl^-$-depleted (B) chloroplasts during the first 3.5 $\mu$s of illumination, as a function of flash number from the dark-adapted state. The extent of the fluorescence yield rise at 3.5 $\mu$s, normalized to the extent of the rise of the first flash, is shown in the insets in Fig. 2. In the uninhibited preparation (Fig. 2A), the extent of

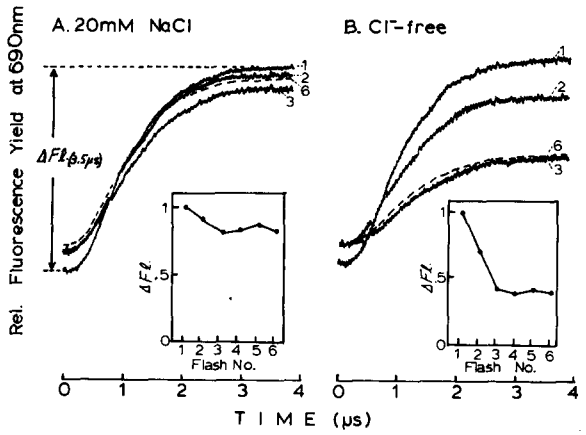



Fig. 2. The effects of chloride depletion on the microsecond rise of the fluorescence yield as a function of flash number. (A) The fluorescence yield in control, dark-adapted chloroplasts. Chloroplasts were incubated in a $Cl^-$-containing medium (0.02 M NaCl/0.05 M Hepes (pH 7.8)/0.1 M $Na_2SO_4$) in the dark for 20 min. In (B) chloroplasts were incubated in a $Cl^-$-free medium (0.1 M $Na_2SO_4$/0.05 M Hepes (pH 7.8)) for 20 min in the dark. Each trace is an average of four experiments, each performed on fresh, dark-adapted samples. The flashes were given 60 ms apart. The fluorescence yield was calculated as shown in Fig. 1 and as described in the text. The chlorophyll concentration was 2.5 $\mu g \cdot ml^{-1}$ in the experiments. Incubations were carried out in the dark at 25 °C. The insets show the extents of the fluorescence rises during the first 3.5 $\mu$s of the flash, normalized to the extent of the first flash.

the fluorescence yield change is relatively high on all flashes and oscillates as a function of flash number with periodicity of four, as has been shown previously [15,20]. The results are different in $Cl^-$-depleted preparations as seen in Fig. 2B. On the first flash, the rate and extent of the fluorescence yield increase are similar to those seen in the $O_2$-evolving samples. There is a slight depression in the extent of the fluorescence increase on the second flash and the level is severely depressed on all subsequent flashes. These results indicate that there are two reducing equivalents on the donor side of PS II in the $Cl^-$-depleted samples, Z, and an additional endogenous donor to Z. Under the conditions of this experiment, $Z^+$ must be reduced by the endogenous donor in less than the 60 ms following the first flash, but much more slowly following subsequent flashes. These results are interesting in comparison to the results obtained when the chloroplasts are subjected to a variety of other treatments which also inhibit $O_2$ evolution, such as Tris-washing, incubation at high pH, or

incubation in high concentrations of $NH_2OH$. Under these conditions, a single endogenous donor to P-680 is observed, Z [13,15,21]. The $Cl^-$-depleted preparation also differs from the Tris- and $NH_2OH$-washed preparations in the rate of the fluorescence yield rise. The latter treatments lead to a substantial slowing in the rate of $P^+$-680 reduction by Z [7,21], while the rate in the $Cl^-$-depleted samples is, within the time resolution of our instrument, as fast as that measured in uninhibited preparations. This is in keeping with the observation that Tris-washing and $NH_2OH$ treatment alter the redox [22,23] and EPR-detected properties [24] of Z. $Cl^-$ depletion appears to modify the electron transfer chain on the donor side of PS II beyond Z, and without affecting Z.

Fig. 3 demonstrates the effect of incubation time in $Cl^-$-free medium on the extent of the fluorescence yield change as a function of flash number. Plotted in Fig. 3A is the change in fluorescence yield (normalized to the extent of the first flash) following 1–6 flashes in chloroplasts incubated in $Cl^-$-free medium for 0.5, 5 and 18 min. The time-course of the inhibitory process is replotted in Fig. 3B, in which the normalized fluorescence yield following the second, third and sixth flashes is shown as a function of incubation time in $Cl^-$-free medium. The most striking result in these figures is the relative insensitivity to the incubation time of the extent of the fluorescence yield change following the second flash. This demonstrates that $Cl^-$ depletion affects the ability of the water-oxidizing complex to re-reduce Z after the second, but not after the first flash. The small inhibition of the change during the second flash may reflect a lowered equilibrium constant for the electron transfer from the endogenous donor to $Z^+$ in the $Cl^-$-depleted chloroplasts. At longer incubation times than those shown here, an inhibition of the change following all flashes was seen, presumably because of nonspecific effects of ageing.

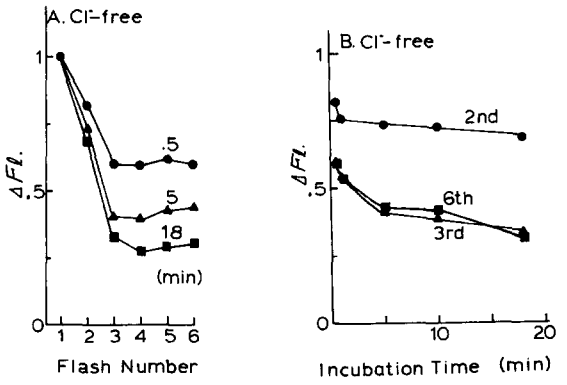



Fig. 3. The effect of incubation time in $Cl^-$-free medium on the fluorescence yield after 1–6 flashes. (A) The effect of incubation time (0.5, 5 and 18 min) in $Cl^-$-free medium on the fluorescence yield during the first six flashes following dark-adaptation. (B) Replotting of the data showing the extent of fluorescence at 3.5 μs following the second, third and sixth flashes after incubation in $Cl^-$-free medium for 0.5–18 min. Experimental conditions were the same as in Fig. 2 except for the length of incubation in $Cl^-$-free medium.

The pattern of inhibition due to $Cl^-$ depletion

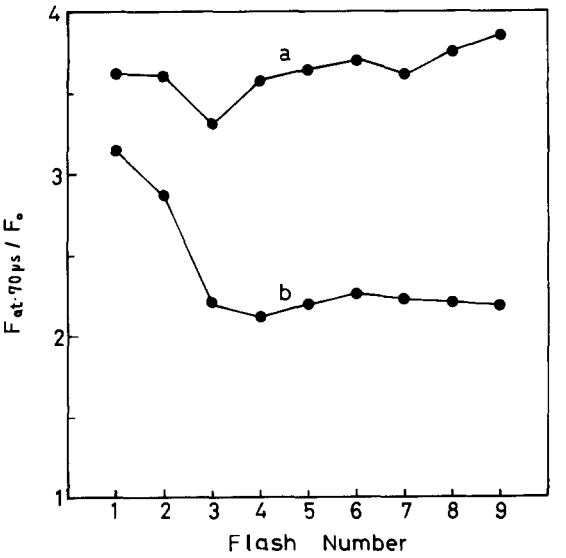



Fig. 4. The effects of $Cl^-$ depletion on the fluorescence yield at 70 μs after flash excitation. The fluorescence yield was measured with a low intensity measuring flash given 70 μs after flashes of saturating intensity using the double flash fluorescence instrument described in Ref. 17. The actinic flashes were fired 100 ms apart. (a) The fluorescence yield of chloroplasts incubated in $Cl^-$-containing (20 mM) medium. (b) The fluorescence yield in chloroplasts incubated in $Cl^-$-free medium for 16 min. All incubations were performed in the dark at 25 °C. The other experimental conditions were the same as in Fig. 2. It should be noted that the differences in the pattern of the change for the first two flashes among Figs. 2, 3 and 4 reflect the different normalization procedures used. In Figs. 2 and 3, the extent of the change for each flash is plotted with reference to the initial fluorescence level for each flash, and the changes have been normalized to the extent of the rise of the first flash. In this figure, the change with reference to the dark fluorescence level ($F_0$) before the group of flashes is shown.

seen when a weak measuring flash was used to monitor the fluorescence level 70 μs after an actinic flash, was the same as that seen when the change was measured at 3.5 μs as demonstrated by Fig. 4. Fig. 4a shows the pattern of fluorescence yield change following a series of actinic flashes in uninhibited preparations. The yield remains relatively high following all flashes and oscillates with period four, as noted in the 3.5 μs fluorescence levels. In Fig. 4b, the pattern observed with $Cl^-$-depleted chloroplasts shows a high fluorescence yield on the second flash but low levels thereafter. Again, this is in agreement with the results at 3.5 μs and with the model in which $Cl^-$-depleted samples retain an endogenous electron donor beyond Z, allowing the donor system to provide two reducing equivalents following dark-adaptation in the absence of exogenous electron donors. The small partial quenching remaining at 70 μs after the second flash (Fig. 4b) may reflect the relatively low equilibrium constant ($K < 10$) for the electron transfer from Z to $P^+$-680, and the fact that $Z^+$ is not rereduced following the second actinic flash in $Cl^-$-depleted chloroplasts.

*Restoration of electron flow to $Z^+$ by $Cl^-$ or exogenous $Mn^{2+}$*

We have also investigated the restoration of electron transport in the oxygen-evolving complex by readdition of chloride, and we have looked at the effect of an exogenous donor, $Mn^{2+}$, on $Cl^-$-depleted samples. These results are shown in Fig. 5. Fig. 5A shows the normalized fluorescence yield following 1–6 flashes in $Cl^-$-depleted samples in the absence and presence of the exogenous electron donor, $Mn^{2+}$ (0.0, 0.1 and 1.4 mM). Addition of the electron donor, $Mn^{2+}$, increased the extent of the fluorescence yield change during the second and all subsequent flashes in the $Cl^-$-depleted chloroplasts. This indicates that $Z^+$, whose re-reduction by the endogenous donor is inhibited after the second flash in $Cl^-$-depleted systems, can be rapidly re-reduced either directly or indirectly by $Mn^{2+}$. The extent to which $Z^+$ could be re-reduced under these conditions varied with the concentration of added $Mn^{2+}$. Relatively high concentrations were required for any substantial restoration of Z. Fig. 5B shows the change in fluorescence yield after 1–6 flashes upon readdition of

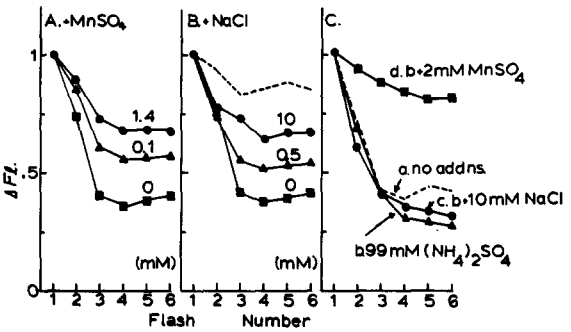



Fig. 5. The effect of addition of $Mn^{2+}$, $Cl^-$ and $(NH_4)_2SO_4$ on the fluorescence yield of $Cl^-$-depleted chloroplasts. (A) The effect of addition of 0.0, 0.1 and 1.4 mM $Mn^{2+}$, a PS II donor, on the fluorescence yield of $Cl^-$-depleted chloroplasts during 1–6 flashes. (B) The effect of readdition of $Cl^-$ (0.0, 0.5 and 10 mM NaCl) on the fluorescence yield of $Cl^-$-depleted chloroplasts. The dotted line shows the fluorescence yield of uninhibited chloroplasts for comparison. (C) The effect of (a) no additions to $Cl^-$-depleted chloroplasts; (b) the addition of 99 mM $(NH_4)_2SO_4$ to $Cl^-$-depleted samples; (c) the addition of 10 mM $Cl^-$ to sample (b) ($Cl^-$-depleted + 99 mM $(NH_4)_2SO_4$); and (d) addition of 2 mM $Mn^{2+}$ to sample (b) ($Cl^-$-depleted + 99 mM $(NH_4)_2SO_4$). All measurements were made 10 min after additions ($Mn^{2+}$, NaCl, $(NH_4)_2SO_4$) were made to $Cl^-$-depleted samples. All other experimental conditions as in Fig. 2.

$Cl^-$ (0.0, 0.5, and 10 mM) to $Cl^-$-depleted preparations. The dotted line shows the fluorescence yield pattern in uninhibited chloroplasts. Readdition of $Cl^-$ had little effect on the extent of the fluorescence yield rise during the first and second flashes but led to an increase during subsequent flashes. This indicates that $Cl^-$ is required for rapid electron donation to $Z^+$ after the second, but not after the first flash. Fig. 5C shows (dotted line, a) the normal pattern seen after $Cl^-$ depletion; (b) the effect of subsequent addition to $Cl^-$-depleted chloroplasts of 99 mM $(NH_4)_2SO_4$; (c) the effect of a further addition of 10 mM NaCl (b + 10 mM NaCl); (d) the pattern after addition of 2 mM $MnSO_4$ to $Cl^-$-depleted, amine-treated chloroplasts (b + 2 mM $MnSO_4$). High concentrations of $NH_4^+$ have previously been shown to inhibit the donor side reactions [25–27]. When the effects of $(NH_4)_2SO_4$ on the fluorescence rise during 3.5 s were measured using uninhibited chloroplasts, only a small inhibition was observed at 99 mM $(NH_4)_2SO_4$, even when the time between flashes was as short as 60 ms (data not shown).

This indicates that re-reduction of $Z^+$ was still able to occur, and was largely complete in 60 ms, after all flashes under these conditions, although re-reduction presumably occurred at an inhibited rate [26]. Addition of $(NH_4)_2SO_4$ to the $Cl^-$-depleted chloroplasts further depressed the fluorescence yield during the fourth and subsequent flashes (Fig. 5C (c)). The recovery of the fluorescence rise during the third flash by readdition of NaCl was inhibited in the presence of $(NH_4)_2SO_4$ (Fig. 5C (d)). Similar antagonistic effects between $NH_4^+$ and $Cl^+$ have been reported previously [2,28] from measurements of steady-state oxygen evolution. The donation to Z observed after addition of the donor, $Mn^{2+}$, was insensitive to the presence of $(NH_4)_2SO_4$ (Fig. 5C (4)), indicating that the recovery of the fluorescence rise after addition of $Mn^{2+}$ involves a different mechanism from that giving rise to the recovery after addition of $Cl^-$.

The results in Fig. 6 show the time-course of the re-reduction of $Z^+$ in $Cl^-$-depleted chloroplasts following the third flash, and the effect on the kinetics of the recovery of addition of (A) $Cl^-$ (0.0, 0.5 or 10 mM), or (B) the exogenous donor, $Mn^{2+}$ (0.0, 0.05 or 1.4 mM). In these experiments, the time-course of reduction of $Z^+$ was measured from the variation of extent of the rapid fluorescence yield change on varying the time between successive flashes in the train. The shortest time between flashes was 60 ms, due to the time taken

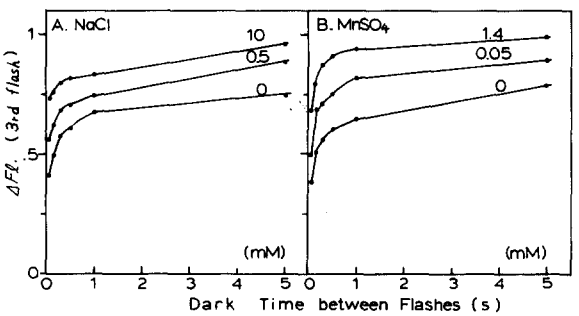



Fig. 6. The effects of addition of NaCl and $Mn^{2+}$ on the dark recovery kinetics of the fluorescence rise during the third flash. The extent of the fluorescence rise during the third flash was measured at different flash frequencies and plotted against the dark time between successive flashes. In (A), NaCl (0.0, 0.5 and 10 mM) was added to the $Cl^-$-depleted chloroplasts before the experiment. In (B), $MnSO_4$ (0.0, 0.05 and 1.4 mM) was added to the $Cl^-$-depleted chloroplasts. All other experimental conditions as in Fig. 2.

to read data from the transient recorder. In the $Cl^-$-depleted preparations, a fraction of $P^+$-680 was rapidly reduced after the third flash, even when the time between flashes was 60 ms, indicating that a fraction of $Z^+$ had been re-reduced in this time after the second flash. The time-course of re-reduction of $Z^+$ from 60 ms onwards was strongly biphasic, with components of approx. 100 ms and more than 10 s. Addition of $Cl^-$ (Fig. 6A) led to a more extensive re-reduction of $Z^+$ during the 60 ms preceeding the first measurement, so that the slower phases contributed a smaller fraction of the total recovery. Addition of $Mn^{2+}$ to the $Cl^-$-depleted preparation (Fig. 6B) also led to an accelerated re-reduction of $Z^+$. In this case, a recovery phase of approx. 100 ms contributed the main component of the re-reduction in the time range after 60 ms, and by extrapolation, could be seen to account for a substantial portion of the total re-reduction.

In order to resolve the kinetics of recovery over the time range up to 60 ms, we have used the double flash instrument (see Fig. 4) equipped with an additional actinic flash lamp, and varied the time between the flash used for measurement and a preceeding flash delivered by the extra lamp. Fig. 7 shows the result of experiments in which the rate of re-reduction of $Z^+$ was measured after the 10th flash of a series, in uninhibited chloroplasts (a); and in $Cl^-$-depleted chloroplast before (b) and after supplementation with $Cl^-$ (c), or $Mn^{2+}$ (d). The reduction time for $Z^+$ in $Cl^-$-depleted chloroplasts (Fig. 7b) showed two relatively slow phases, one of 100 ms and another of many seconds. These correspond to the slow phases seen after the third flash with the 3.5 $\mu$s instrument (Fig. 6A), although in the latter case some residual, more rapid, kinetically unresolved phase was also present. This rapid phase could be attributed to the fraction of centers in the state $S_0$ before the flash train. In these centers, re-reduction of $Z^+$ would still have occurred rapidly after the second flash. For the change measured after ten flashes, all centers would be exhausted of endogenous donors, and the true (inhibited) kinetics of re-reduction of $Z^+$ should be revealed. In uninhibited chloroplasts, the reduction of $Z^+$ proceeded with a half-time of about 450 $\mu$s, with no obvious slow phase (Fig. 7a). Since the measurement was made

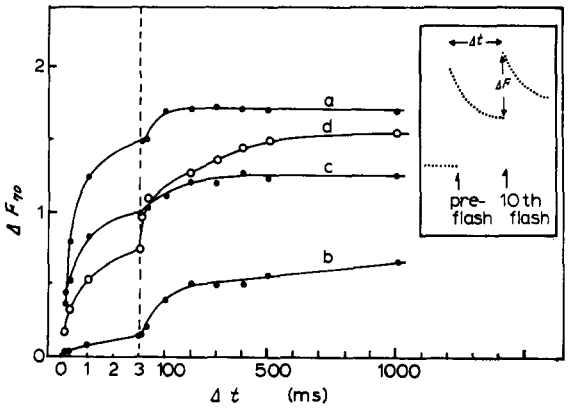



Fig. 7. The dark recovery of Z measured by the increase of fluorescence yield 70 $\mu$s after the tenth actinic flash. For delay times ($\Delta t$) of 100 ms or less, an additional flash of light (pre-flash) was given at variable times preceeding the tenth actinic flash given to a sample, and the increase of the fluorescence yield induced by the tenth flash was plotted against the delay time between the pre-flash and the tenth flash. The fluorescence yield at the time of the firing of the pre-flash was estimated from the decay kinetics of the fluorescence change following the ninth flash. For delay times ($\Delta t$) greater than 100 ms, no pre-flash was given. The increase of the fluorescence yield induced by the tenth flash was plotted against the time between successive flashes. The time-course of the recovery of the high fluorescence state in uninhibited chloroplasts is shown in (a), the recovery in $Cl^-$-depleted systems is shown in (b), the effect of addition of the PS II donor, $Mn^{2+}$, on the recovery is shown in (c), and the effect of readdition of 10 mM NaCl on the recovery of the high fluorescence state is shown in (d). For delay times ($\Delta t$) of less than 100 ms, actinic flashes were given at 100-ms intervals. For delay times ($\Delta t$) 100 ms or longer, the flash rate is the same as the delay time plotted in the figure. All other conditions as in Fig. 2.

after ten preceeding flashes, the S-states would have been almost completely scrambled, and this half-time must represent a composite of the different re-reduction phases due to the different transitions of the S-states [6,29,30]. The time measured is comparable to previous estimates of the recovery of the donor side under steady-state conditions [6]. When the depleted preparations were supplemented with $Cl^-$ (Fig. 7c), a rapid phase of re-reduction of $Z^+$ (half-time 450 $\mu$s) was restored in about 74% of the centers, and the remaining centers recovered slowly. Addition of excess $Mn^{2+}$ also resulted in a rapid re-reduction of $Z^+$, although in this case, an intermediate phase of reduction accounting for 25% of the recovery was present, with a half-time in the range of 100 ms, as well a a very slow phase (Fig. 7d). The presence of the rapid phase of recovery of Z suggests that exogenous $Mn^{2+}$ may be able to reduce the physiological donor to $Z^+$ in the time range between the main flashes of the sequence (100 ms).

*Site of inhibition on $Cl^-$ depletion*

In order to determine the relation between the inhibition observed on $Cl^-$ depletion and the reactions of the oxygen-evolving complex, we have investigated the effects of addition of hydroxylamine at low concentration on the pattern of fluorescence as a function of flash number. In oxygen-evolving preparations, $NH_2OH$ (and $NH_2NH_2$) has been shown to delay the advancement of the S-states by two flashes, leading to a maximal yield of $O_2$ after the fifth rather than the third flash of a series [30,31]. Radmer [32–34] has studied the mechanism in some detail, and has proposed that $NH_2OH$ is oxidized by the complex in its normal dark state ($S_1$), resulting in a modified state ($S_0$ with bound $NH_2OH$) which is converted back to the normal dark state by two oxidation steps linked to electron donation to $P^+$-680 following two flashes. Theg and Jursinic (personal communication, and Ref. 35) have previously shown that $NH_2OH$ at low concentration delays by two flashes the formation of a stable $P^+$-680 in $Cl^-$-depleted preparations. They measured the presence of $P^+$-680 by the appearance of centers showing a high intensity of delayed fluorescence. In $NH_2OH$ – supplemented preparations, this state appeared after five flashes, rather than after three flashes in unsupplemented, $Cl^-$-depleted preparations. We have confirmed and extended these observations using the 3.5 $\mu$s fluorescence yield measurements, as shown in Fig. 8. In the presence of 50 $\mu$M $NH_2OH$, a rapid re-reduction of $P^+$-680 was seen after the first four flashes, and a lower level of fluorescence only after the fifth flash. This indicates that in the presence of hydroxylamine, $Z^+$ was rapidly reduced by endogenous donors, contributing a pool of three equivalents per center. A similar effect was seen when hydrazine was used instead of hydroxylamine, except that rather higher concentrations of the inhibitor were required. The extent of the fluorescence yield change at 3.5 $\mu$s is plotted as a function of flash number for $Cl^-$-depleted and res-

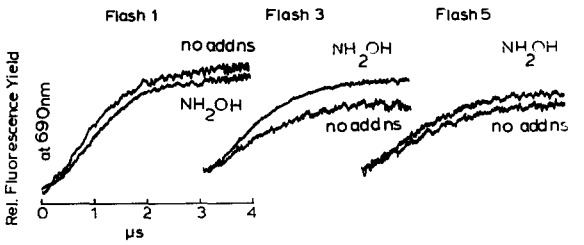



Fig. 8. The effect of addition of $NH_2OH$ on the fluorescence yield during the first, third and fifth flashes of $Cl^-$-depleted chloroplasts. The normalized fluorescence yields in $Cl^-$-depleted chloroplasts in the absence (no addns) and presence of 50 μM $NH_2OH$ during the first, third and fifth flashes after dark adaptation are shown. All other experimental conditions as in Fig. 2.

tored preparations, and for $Cl^-$-depleted chloroplasts supplemented with a range of $NH_2OH$ or $NH_2NH_2$ concentrations, in Fig. 9. At higher concentrations of the reagents, some re-reduction of $Z^+$ occurred after the fifth and subsequent flashes, presumably due to nonspecific donation. In terms of Radmer's mechanism [32], the pattern of inhibition and restoration observed would indicate that the transitions of the S-states from $S_0$ to $S_2$ were unaffected by $Cl^-$ depletion, but that the oxygen-evolving apparatus was unable to advance beyond the $S_2$ state. We have also looked at the effect of addition of low concentrations of $NH_2OH$ (50

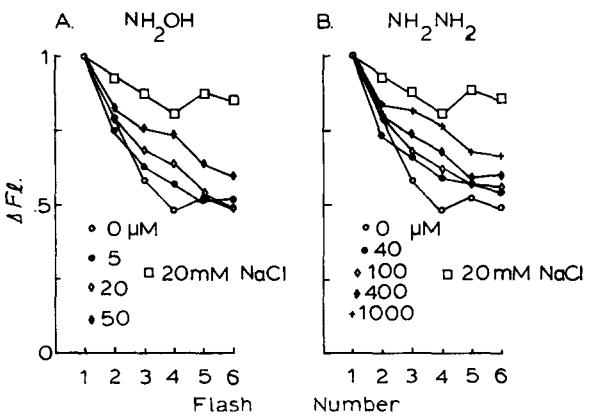



Fig. 9. A titration of the effects of $NH_2OH$ and $NH_2NH_2$ on the fluorescence yield of $Cl^-$-depleted chloroplasts. (A) The effect of $NH_2OH$ (0.0, 5, 20 μM) on the fluorescence yield of $Cl^-$-depleted chloroplasts 3.5 μs after actinic flashes. (B) The effect of $NH_2NH_2$ (0.0, 40, 100, 400 and 1000 μM) on the fluorescence yield of $Cl^-$-depleted chloroplasts 3.5 μs after actinic flashes. Also shown are the fluorescence levels in uninhibited samples. All other conditions as in Fig. 2.

μM) to Tris-washed chloroplasts (data not shown). During the first flash, we see a lower rise in the fluorescence yield than that seen in uninhibited or $Cl^-$-depleted samples and which is independent of the presence or absence of $NH_2OH$. On all subsequent flashes, the fluorescence yield is low and virtually indistinguishable in the presence and absence of $NH_2OH$. At these low concentrations, we can, therefore, eliminate the possibility that $NH_2OH$ is acting as a nonspecific donor to PS II. This reinforces the proposal that in $Cl^-$-depleted systems, $NH_2OH$ is being oxidized specifically by the lower transitions of the S-states, which are inoperable in Tris-washed chloroplasts.

*Redox components of the donor side of PS II, and the nature of the S-states*

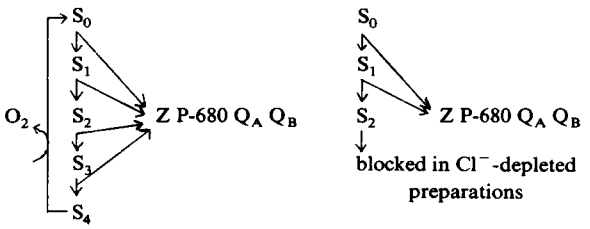
We have demonstrated above that $Cl^-$ depletion inhibits some of the reactions of the system which donates electrons to Z. The S-state model of Kok et al. [29] provides a framework for discussion of these reactions. However, the S-state nomenclature has in the past been used with some ambiguity. When first introduced, the different states were phenomenologically defined with respect to the behavior of the system as a whole. With increasing sophistication in our knowledge of the mechanism of $O_2$ evolution, the S-states have been understood as describing the redox transitions of the components on the donor side of Photosystem II, but without any clear recognition of the chemistry involved in these transitions. In view of our present results, it seems more appropriate to define the S-states in terms of the transitions of the complex donating electrons to $Z^+$.

It is clear that oxidation and reduction of the couples $P^+$-680/P-680 and $Z^+/Z$ normally involve a single electron, and that under physiological conditions, both components are almost completely re-reduced after each flash of a sequence from the dark state [6,36]. It is therefore clear that neither component takes part in the storage of oxidizing equivalents required for oxygen evolution. It has previously been shown that the rate of electron donation to $Z^+$ varies with flash number [6]. This implies that the state of the immediate donor changes with S-state, and suggests that there is no common one-electron couple through which

the oxygen-evolving complex passes electrons to $Z^+$. The results reported here provide strong support for this latter view. In $Cl^-$-depleted preparations, an extra equivalent of reductant is available in the donor pool. This could possibly come from an intermediate donor (D), with $Cl^-$ depletion acting to disconnect the oxygen-evolving complex from a chain consisting of:

$$\text{OEC} \ldots \underset{\text{no } Cl^-}{\uparrow} \ldots \text{D Z P}$$

However, the effects of hydroxylamine and hydrazine at low concentrations have been shown to reflect a specific binding to the oxygen-evolving complex [29–34]. If the simple model above were correct, the $Cl^-$-depleted system would not be able to advance the hydroxylamine- or hydrazine-inhibited systems, and these reagents would have no effect. We can therefore eliminate the notion of an intermediate donor and represent the uninhibited and $Cl^-$-depleted systems as:




Theg and Jursinic (personal communications; see also Ref. 35) have independently arrived at similar conclusions. Although the inhibitory effect of $Cl^-$ depletion throws no light on the nature of the chemical intermediates of the oxygen-evolving complex, our own results, together with recent observations from other laboratories, do allow us to arrive at some specific conclusions.

(a) The EPR-saturation of signal $II_f$ ($Z^+$) is modified in chloroplasts or PS II preparations after Tris-washing or treatment with $NH_2OH$ at mM concentrations, but not by $Cl^-$ depletion or high concentrations of $NH_4^+$ ion [8]. The former, but not the latter, treatments lead to loss of Mn [8,37]. It has been suggested that the high power saturation of $Z^+$ in uninhibited (or $Cl^-$-depleted) systems reflects an interaction with a neighboring Mn center [24,27].

(b) The redox properties [22,23] and kinetics of oxidation of Z [7] are also modified by Tris-washing or by treatment with high concentrations of $NH_2OH$.

(c) The hyperfine structure of an EPR signal associated with the state $S_2$ [38,39] is modified in $Cl^-$-depleted systems [40]. The appearance and disappearance of the EPR signal in uninhibited systems are ascribed to redox changes of a Mn dimer or tetramer [38,41].

(d) From our present results, there is no intermediate electron transfer component between Z and the oxygen-evolving complex. It seems highly probable, therefore, that the electron donor to $Z^+$ is a Mn atom of the complex, and it also seems reasonable to equate this Mn with the one which perturbs the properties of $Z^+$ [24].

(e) Oxidation of a Mn cluster is responsible for at least two of the four oxidizing equivalents accumulated by the oxygen-evolving complex. The oxidation step associated with the disappearance of the hyperfine signal, the transition from $S_2$ to $S_3$, requires $Cl^-$. A possible mechanism relating this $Cl^-$ requirement to electron transfer among bound Mn atoms has been proposed [28].

(f) The S-states can now be associated specifically with transitions of the oxygen-evolving complex, and two of the transitions can be ascribed to identified redox reactions of a Mn complex, even though these are not yet chemically defined.

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