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STUDIES ON THE MECHANISM OF CHLORIDE ACTION ON PHOTOSYNTHETIC WATER OXIDATION

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In this paper, we describe experiments which were designed to probe the mechanism through which Cl^- anions exert their influence on electron transport on the oxidizing side of Photosystem II (PS II). We asked whether photosynthetically active Mn was released from and reinserted into the water-splitting enzyme upon Cl^- removal and subsequent repletion, and obtained evidence suggesting that it was not. To locate the site of the Cl^--dependent lesion, we counted the number of electrons that were still available in Cl^--free chloroplasts for rapid reduction of P-680$^+$ following a flash, and compared our results with other, previously characterized methods of inhibition. Using both delayed and prompt fluorescence as measures of the lifetime of P-680$^+$, we found that Cl^--depleted thylakoids could deliver two electrons to the oxidized PS II reaction center. This is interpreted as indicating that two oxidizing equivalents can be generated and transiently stored by PS II after Cl^- removal. Two alternative schemes which describe the functional location of electron carriers in this portion of the electron transport chain are proposed to account for our data. An experiment designed to distinguish between them is discussed. We also investigated the stability of oxidants produced by the Cl^--depleted PS II. The apparently contradictory results obtained by prompt fluorescence and luminescence measurements are tentatively resolved by postulating the existence of two pathways through which closed reaction centers reopen, only one of which proceeds via a luminescence-producing recombination mechanism. It is suggested that deactivation of the PS-II oxidizing equivalents through both pathways is accelerated by Cl^- removal.

Introduction

It has long been recognized that Cl^- anions are required for maximal rates of electron transport in isolated chloroplasts [1–3]. Fluorescence studies [4–6] and studies of partial reactions using artificial electron donors and acceptors [7–9] have provided convincing evidence that the Cl^--requiring step is on the oxidizing side of PS II. We [10] showed previously that symptoms of severe Cl^- deficiency can be demonstrated regardless of the pH of the suspension medium or the coupling state of the chloroplasts (see Ref. 3), thereby eliminating the possibility that the Cl^- requirement is an artifact of 'damaged' chloroplasts [11].

A number of published experiments have indicated that Cl^- may interact directly with the putative Mn-containing water oxidase [12]. Izawa and

Abbreviations: Chl, chlorophyll; DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; Tes, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}ethanesulphonic acid; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

his co-workers [8,13,14] and Coleman et al. [15] found that when Cl^- is omitted from the suspension medium, this enzyme is more susceptible to inhibition by procedures which are known to solubilize functional Mn. Additionally, Muallem and his collaborators [14,16,17] reported that Cl^- depletion resulted in stabilization of Kok's 'S-states' [18] against deactivation.

In the experiments reported in this paper, we have reinvestigated the effects of Cl^- deficiency on the water-oxidizing apparatus. In agreement with results obtained by Muallem and his co-workers [14,17,19], we found that under conditions of reversible inactivation due to Cl^- removal, the oxidizing side of PS II was able to accumulate two oxidizing equivalents, transiently forming the S_2 state. In contrast to those authors [14,16,17], however, our data suggest that those stored oxidants are more susceptible to deactivation when Cl^- is withheld from the reaction medium. We also obtained evidence suggesting that the irreversible photoinactivation of Cl^--free chloroplasts [3,9] is accompanied by a shift in the site of inhibition from an S-state transition of the water-splitting enzyme to the reduction of P-680$^+$ by the secondary electron donor, Z (for a review of the organization of PS II see Ref. 20).

Materials and Methods

Stock chloroplasts were prepared in the absence of Cl^- from 2–4-week-old pea seedlings (*Pisum sativum*, var. Progress 9) or 6–10-week-old spinach plants (*Spinaceae oleacea L.*) as detailed in Ref. 10. Briefly, leaves were homogenized and washed twice in 200 mM sucrose/25 mM sodium-Hepes (pH 7.5)/5 mM $MgSO_4$. They were resuspended in identical medium at either pH 7.5 or 7.0, diluted to 70% of their original concentration with ethylene glycol and then stored frozen in liquid nitrogen for less than 2 months. Tris-washed chloroplasts were prepared as in Ref. 21, while NH_2OH-incubated samples were prepared in the light as in Ref. 22. Measurements of chloroplast activities were performed in alkaline assay media described in the figure legends. As shown previously [10], chlorplasts prepared in Cl^--free media as outlined above retain essentially the full complement of functional Cl^- in the PS II Cl^- site. Therefore, in every experiment related to the PS II Cl^- requirement, an uncoupling concentration of either gramicidin D or nigericin was added to facilitate the removal of this residual functional Cl^- from the water-oxidizing enzyme [10].

Steady-state rates of oxygen evolution were assayed in a Clark-type oxygen electrode and oxygen flash yields were determined with a bare-platinum electrode [23]. The prompt and delayed fluorescence experiments shown in Figs. 5 and 6 were performed in a 90° set-up similar to that described earlier [24]. For the latter, a shutter placed between the sample and photomultiplier tube was opened 50 ms after the actinic light was extinguished. Blue (Balzers DT Blau) and red (Schott 682 narrow-band pass for prompt fluorescence and Schott RG 663 for luminescence) filters were placed before the actinic light and the photomultiplier tube, respectively.

The prompt and delayed fluorescence experiments of Figs. 2–4 were performed in a different apparatus which has been described elsewhere [25]. A Corning CS4-96 glass filter was placed between the Xenon flash lamp and the sample, while a Corning CS2-64 filter was placed between the sample and the photomultiplier tube. The photomultiplier was switched off during the excitation flash with an electronic gating circuit (cut-off approx. $5 \cdot 10^3$). Prompt fluorescence was probed with a weak, nonexciting flash delivered 80 μs after an excitation flash of saturating intensity.

Results and Discussion

Functional Mn is not released from the reversibly inhibited, Cl^--free water-splitting enzyme

It has recently been shown (Ref. 26 and Theg, S.M., unpublished observation) that when chloroplasts were partially depleted of Cl^- in a SO_4^{2-}-containing medium, the resulting lower Hill activity was not accompanied by a change in the turnover time of the electron transport chain. This demonstrates that Cl^- depletion causes complete, rather than partial, inhibition of those electron transport chains effected. Many other treatments that apparently result in the complete inhibition of individual electron transport chains cause the release of functional Mn from the water-splitting enzyme complex [12]. Yamashita et al. [27] were

the first to show that reactivation of Tris-washed chloroplasts after the assumed release of Mn is sometimes possible in the dark and, surprisingly, does not require externally added Mn^{2+}. This puzzling observation was explained in part by Blankenship et al. [28], who demonstrated that the Mn^{2+} released by Tris-treatment remained trapped by the thylakoids and was available for reinsertion into the water-oxidizing enzyme upon dark reactivation. It seemed possible, therefore, that a similar Mn release and reinsertion mechanism was responsible for the reversible inhibition observed during Cl^- depletion and subsequent repletion.

As demonstrated in a different context [29], the divalent cation ionophore, A23187, is able to carry Mn^{2+} across the thylakoid membrane (but see Ref. 30). If the Mn release and reinsertion mechanism described above did in fact operate, then ionophore A23187 could be expected to block the recovery of Hill activity upon Cl^- repletion by carrying the released Mn^{2+} out of the thylakoid and away from its reinsertion site. Hence, we tested the ability of ionophore A23187 to render the Cl^--dependent inhibition of electron transport irreversible.

The traces a–d in Fig. 1 demonstrate that Cl^- depletion did occur during a 2-min dark incubation of chloroplasts with an uncoupler in alkaline Cl^--free assay medium (cf. Ref. 10), and that 5 μM ionophore A23187 did not block this process. An unexplained 36% inhibition of the control, plus Cl^- sample was repeatedly observed. Trace e shows that when Cl^- was added after a 2-min Cl^--free incubation, (i.e., when Cl^- was added to chloroplasts in an 80% inhibited state; see trace b), 81% of the initial Hill activity was recovered. In trace f, the recovery of Hill activity upon Cl^- addition was 55% complete when ionophore A23187 was included in the reaction medium. Considering both the lack of complete recovery in the absence of ionophore A23187 and the inhibition caused by the ionophore, this 55% recovery represents the maximum that can be expected under the conditions of trace f. In order to maximize the interaction between ionophore A23187 and any released Mn^{2+}, this experiment was performed without the usual complement of Mg^{2+}. Addition of 2 mM sodium-EDTA to the reaction vessel did not affect the results (not shown). In a separate experiment

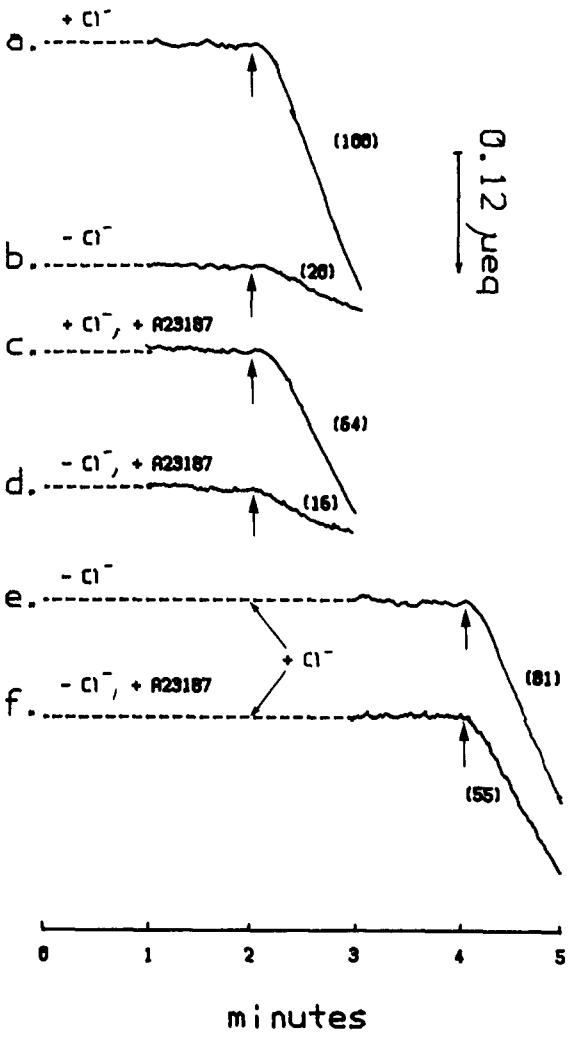

Fig. 1. The lack of effect of ionophore A23187 on the loss and restoration of Hill activity upon Cl^- removal and subsequent repletion. Traces a and b, 2-min dark incubation in the presence and absence of 10 mM NaCl, respectively, before the rate of the Hill reaction (water to methyl viologen) was assayed. Traces c and d, as in a and b, except 5 μM A23187 was present. Trace e, 2-min dark incubation without Cl^-, then 10 mM Cl^- added at $t = 2$ min and electron transport rate assayed at $t > 4$ min. Trace f, as in e, except 5 μM ionophore A23187 was present. Bold vertical arrows indicate light on; the skewed arrows in traces e and f show the time of Cl^- addition. Assay medium contained 25 mM sodium-Hepes (pH 8.1), 5 mM K_2SO_4, 1 μM nigericin, 0.1 mM methyl viologen and 50 μg Chl/ml. The percent remaining Hill activities are given by the numbers in parentheses and represent the average of two determinations; 100% corresponds to a rate of 402 μequiv./mg Chl per h.

with the same batch of ionophore and chloroplasts, we determined that 5 μM ionophore A23187 halved the extent to which the thylakoids could accumulate protons in the light, and nearly tripled the normalized initial rate of proton efflux after the light was extinguished (data not shown). These results, which show that the ionophore was working properly under the conditions of the experiment depicted in Fig. 1, indicate that the reversible lesion in PS II electron transport caused by Cl^- depletion is not due to the release and reinsertion of photosynthetically active Mn.

Identification of an S-state transition as the locus of inhibition in Cl^--depleted chloroplasts

The site of electron transport inhibition in Cl^--depleted chloroplasts is located on the oxidizing side of PS II [4–9], just like the lesion caused by treatments such as Tris-washing. Several authors [21,31,32], whose works and interpretations were recently discussed in depth by Bouges-Bocquet [20], have used prompt fluorescence and μs luminescence to pinpoint the site of Tris-induced inhibition even further, and we have used these techniques to attempt to locate the Cl^--dependent lesion in an analogous fashion.

$P\text{-}680^+$ is known to be a quencher of chlorophyll *a* fluorescence [33]. It is reduced in less than 1 μs in control chloroplasts [20], and in less than 10 μs in Tris-washed thylakoids at pH 7.5 [34]. An analytical flash of low intensity given 80 μs after an actinic flash will detect high chlorophyll *a* fluorescence because $P\text{-}680^+$ will be reduced in this time, whereas Q_a^-, with a reoxidation time of 200 μs or greater [35] will still be largely reduced. In control samples, the fluorescence would remain high after every flash, because electrons are available from the oxidation of water for $P\text{-}680^+$ reduction. In chloroplasts that are inhibited on the water-side of P-680, electrons will not necessarily be available on every flash. Instead, when the reducing capacity of this portion of the electron

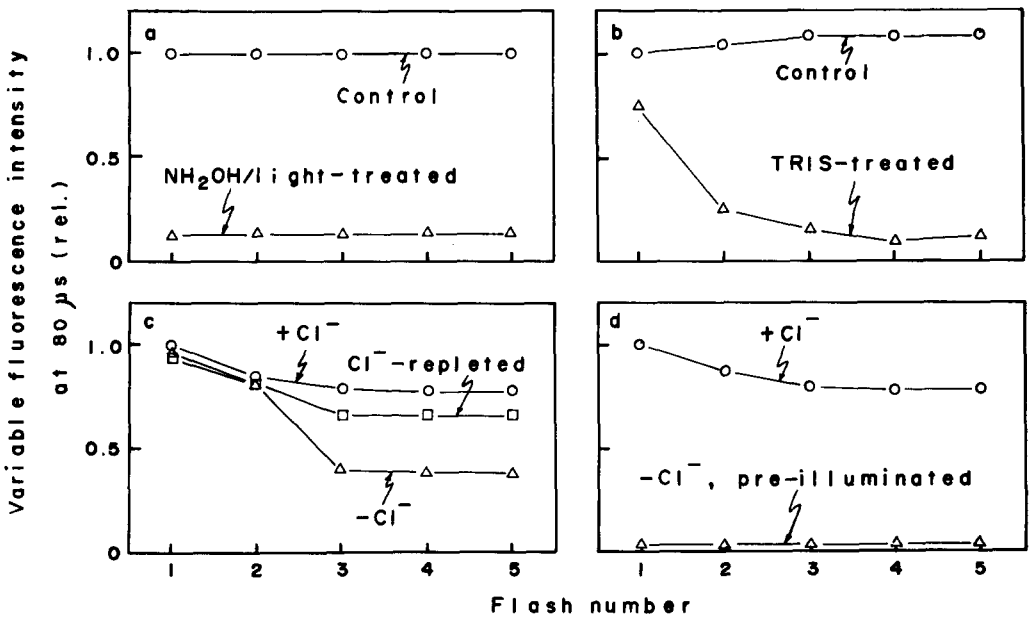

Fig. 2. Effects of inhibition of water oxidation by various means on the flash number dependence of variable fluorescence yield 80 μs after a flash. Variable fluorescence was calculated as $[F(80\ \mu s) - F_0]/F_0$, where F_0 is the fluorescence level determined before the actinic flash and $F(80\ \mu s)$ is the fluorescence level 80 μs after the flash. The flash frequency was 10 Hz. For panels (a) and (b), the assay medium contained 400 mM sucrose, 50 mM sodium-Tes (pH 7.5), 10 mM NaCl and 5 mM $MgSO_4$. For panels (c) and (d), assay medium contained 25 mM sodium-Hepes (pH 8.1), 5 mM $MgSO_4$, 0.5 μM gramicidin, 10 μg Chl/ml, and when present, 10 mM KCl. Cl^- depletion was achieved by a 1.5-min incubation in the assay medium in the absence of Cl^-. For panel (d), photoinactivated samples were prepared by illuminating Cl^--free chloroplasts for 30 s with intense white light, followed by dark readaptation for 3 min.

transport chain is exhausted, P-680$^+$ will not be rapidly reduced after the flash and chlorophyll *a* fluorescence will remain quenched. The number of flashes delivered before the fluorescence yield drops is, therefore, a measure of the number of electrons still available for rapid P-680$^+$ reduction after inhibition.

Fig. 2 shows the flash number dependence of 80 μs variable fluorescence from chloroplasts which were inhibited by four different methods. In panel a, thylakoids were washed with NH_2OH in the light, a treatment which is thought to block electron flow between Z and P-680 [22,36,37]. As expected from samples with such a lesion, no electrons were available for P-680$^+$ reduction, and consequently, fluorescence was low on all flashes. In the Tris-washed sample (panel b), the 80 μs fluorescence intensity was high on the first flash, but significantly lower on the second and subsequent flashes, indicating that one electron was still available for P-680$^+$ rereduction. This is in agreement with experiments performed by others (Refs. 21,31,32,34; cf. Ref. 20) and is consistent with the placement of the Tris-induced lesion between Z (or Z_1, Ref. 20) and the next, higher potential electron donor.

Panel c shows that fluorescence did not reach a low level in Cl^--depleted chloroplasts until the third flash. This indicates that P-680$^+$ can be rapidly rereduced twice in Cl^--free samples, and that the site of Cl^--dependent lesion is one step further removed from the PS II reaction center than is the Tris-induced block. The Cl^--repleted sample was similar to, but not identical with, the control, probably as a result of incomplete recovery of the initial Hill activity (cf. Fig. 1).

An interesting situation was encountered in the experiment of panel d in Fig. 2, which shows a low fluorescence level regardless of the flash number for Cl^--deficient thylakoids that had been exposed to light. It is known [3,9] that Cl^--depleted chloroplasts become irreversibly inactivated after illumination, as indicated by the inability of subsequent Cl^- addition to restore Hill activity. The obtained data resemble that seen in samples treated with NH_2OH in the light and suggest that photoinactivation of Cl^--free chloroplasts causes a new lesion which appears to be located closer to P-680 than that caused by simple Cl^- removal. This

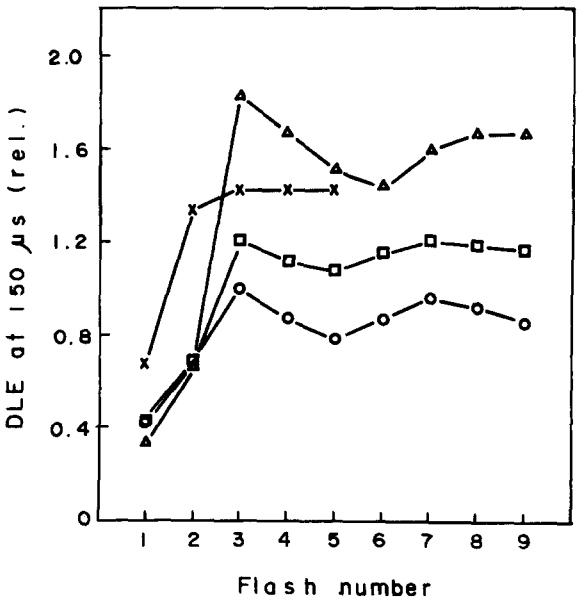

Fig. 3. The effects of Tris-washing and Cl^- depletion on delayed light emission (DLE) at 150 μs, after a flash as a function of flash number. ○———○, control chloroplasts; △———△, 1.5 min dark incubation without Cl^-; □———□, 3.0 min dark incubation, 1.5 min without Cl^-, then 1.5 min after addition of Cl^-; ×———×, Tris-washed chloroplasts. Assay medium contained 40 mM sucrose, 50 mM potassium-Tes (pH 8.1), 5 mM $MgSO_4$, 0.5 μM gramicidin and 10 μg Chl/ml; when present, KCl concentration was 10 mM. Flash repetition rate was 2 Hz and all values were normalized to that of the control on the third flash. All samples were dark-adapted as stock suspensions on ice for at least 15 min.

interpretation is borne out by artificial electron donor studies and will be the subject of a future publication.

Delayed light intensity emitted from normal, isolated chloroplasts 150 μs after a saturating flash is known to oscillate with a period of 4 in phase with oxygen flash yields [38]. When the water-splitting complex is perturbed, for example, by Tris washing, this component of the delayed light emission becomes comparatively higher as the lifetime of P-680$^+$ is increased. Thus, the number of flashes delivered to an inhibited sample before delayed light rises indicates the number of electron donors to P-680$^+$ residing between the inhibition site and the reaction center.

The results of one such experiment are shown in Fig. 3. It can be seen that the usual period 4 oscillation of the 150 μs delayed light emission in

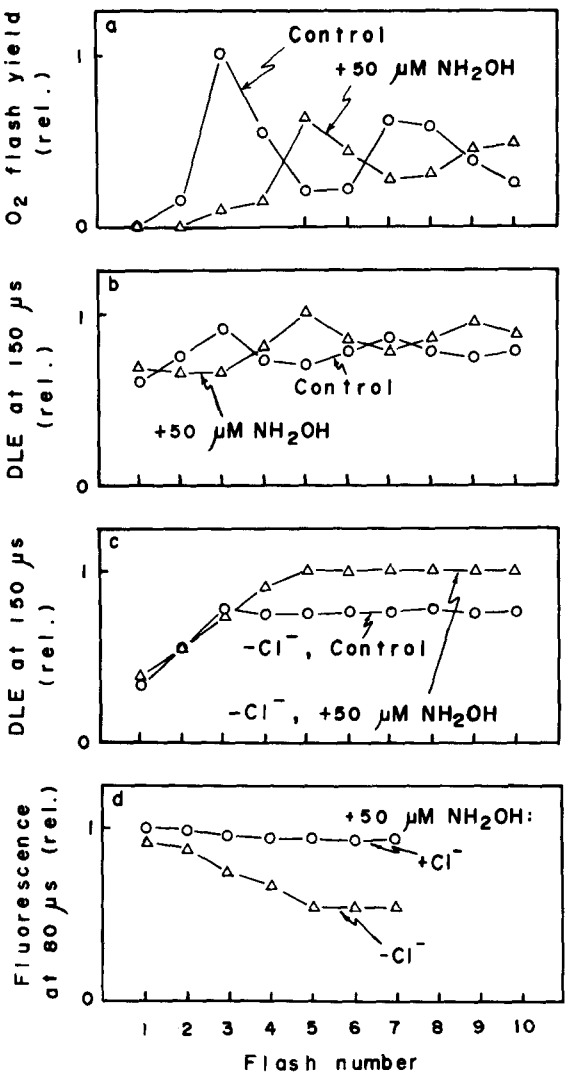

Fig. 4. The effect of 50 μM NH_2OH on the oxygen flash yield pattern and on the flash number dependence of prompt and delayed fluorescence after a flash in the presence and absence of Cl^-. (a) Oxygen flash yields were measured with a rate electrode at a Chl concentration of 500 μg/ml. 50 μM NH_2OH was added to this sample, and flashes were delivered after at least 10 min dark adaptation. (b) 50 μM NH_2OH was added to thylakoids at a Chl concentration of 500 μg/ml. After at least 10 min dark adaptation, the sample was diluted to 10 μg Chl/ml and immediately assayed for delayed light emission (DLE). (c) Same as (b) but thylakoids depleted of Cl^- as in Fig. 3c. (d) NH_2OH addition as in (b) but measurements performed as in Fig. 3c.

control or Cl^--repleted chloroplasts was replaced by a somewhat higher delayed fluorescence intensity after Cl^- depletion or Tris-washing. The flash number dependence of the rise in delayed light emission supports the results shown in Fig. 2b and c, i.e., one and two electron donors apparently are available for P^+-680 reduction after Tris- and Cl^--dependent inhibition respectively.

The data of Figs. 2 and 3 indicate that two electrons were available for rapid P-680$^+$ reduction in reversibly inhibited Cl^--free samples. To test whether S-state transitions of the oxygen-evolving system were involved in the donation of these electrons, we examined the effect of NH_2OH on the flash number dependence of delayed light emission and 80 μs fluorescence in Cl^--free chloroplasts. Bouges-Bocquet [39] discovered that through interactions with the S-states, low concentrations of this compound delay the normal flash pattern of O_2 evolution by two flashes. Since such low NH_2OH concentrations should not permit direct electron donation to Z^+, we reasoned that an effect on luminescence or 80 μs prompt fluorescence from Cl^--depleted chloroplasts would indicate that the electrons used for rapid P-680$^+$ reduction originated in the water-splitting apparatus.

Panel a of Fig. 4 shows that the oxygen flash yield pattern obtained with Cl^--sufficient chloroplasts in a rate electrode was indeed delayed by two flashes by the addition of 50 μM NH_2OH. Similar results were observed when the flash pattern was measured by monitoring the 150 μs component of delayed light emission (panel b). The luminescence experiment with Cl^--depleted samples is shown in panel c, where it can be seen that although NH_2OH did not alter the delayed light pattern during the first three flashes, it did cause a continuing rise of the luminescence intensity until the fifth flash, thereby delaying the attainment of the highest intensity by two flashes. Similar results were obtained when the number of electrons available for P-680$^+$ reduction were determined by monitoring the 80 μs prompt fluorescence signal (panel d). Provided we are correct in interpreting the effects of NH_2OH on the luminescence and prompt fluorescence flash patterns to be a consequence of its interactions with the S-states, our results, although somewhat ambiguous, are compatible with the assumption that the S_2 state can be formed in Cl^--depleted samples.

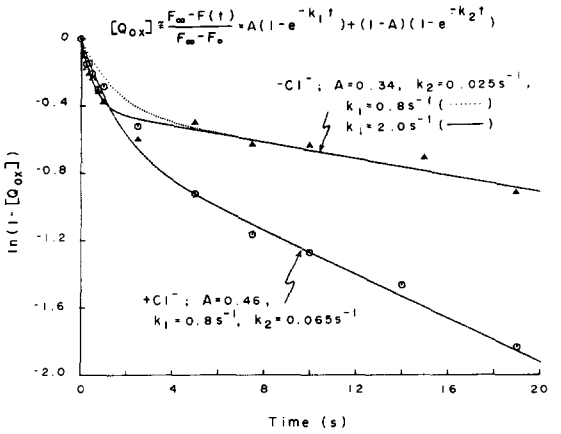

Fig. 5. Semilogarithmic plot of the time-course of the return to the low fluorescence state in the dark after an illumination period. O——O, Control, Cl^--sufficient chloroplasts; △——△, Cl^--deficient chloroplasts. Chloroplasts (10 μg Chl/ml) were added to a medium containing 200 mM sucrose, 25 mM sodium-Tricine (pH 8.0) (once recrystalized to remove Cl^-), 5 mM $MgSO_4$, 0.1 μM gramicidin and when present, 10 mM NaCl. After 5.5 min in the dark, 1 μM DCMU was added. At $t = 6$ min, the sample was preilluminated for 9 s with blue light, darkened for the time indicated on the abscissa, then reilluminated, at which time the initial level of fluorescence was recorded. The long Cl^- depletion time was required because those chlorplasts were prepared and stored in the presence of Cl^-. The actinic light was intense enough (300 μW/cm²) to close all the reaction centers during the 9-s preillumination period, and all values of $F(t)$ were normalized to the maximal fluorescence levels for each sample. The lines were calculated according to the biphasic kinetic equation shown in the figure using the indicated parameters; for the Cl^--depleted sample, the solid line was drawn using $k_1 = 2.0\ s^{-1}$ and the dotted line using $k_1 = 0.8\ s^{-1}$.

Stability of the higher S-states of the Cl^--free water-splitting complex

Since delayed light emission is one expression of the recombination of the electron-hole pair produced by the photoact of the PS II reaction center, the intensity of the emission can be used as a measure of the rate of this back-reaction. In view of the experiments of Muallem and his co-workers [14,16,17] which suggested that Cl^- depletion stabilizes the higher S-states of the water-splitting enzyme, it is somewhat surprising that in our experiments we observed higher luminescence intensities from thylakoids after removal of Cl^-. We therefore reinvestigated the effect of Cl^- depletion on the stability of the PS II oxidants against deactivation.

In DCMU-poisoned chloroplasts, the initial level of prompt fluorescence measured upon illumination ($F(t)$) is a measure of the extent of Q_a reduction, i.e., $F(t)$ is lowest in dark-adapted chloroplasts with fully oxidized Q_a and somewhat higher in samples that had been recently exposed to light. The rate at which $F(t)$ returns to its lowest level (F_0) after a light period serves as an approximate measure of the rate of the reoxidation of Q_a^- through the back-reaction [40].

Fig. 5 shows a semilogarithmic plot of the time-course of Q_a^- reoxidation obtained in such an experiment. In both the presence and absence of Cl^-, the reoxidation kinetics were clearly not monophasic, nor could they be consistently interpreted as second-order, as reported by Bennoun [41] (analysis not shown). Because data points during the early stage of the rapid phase could not be accurately determined, kinetic analysis was performed by curve fitting. The solid lines in the figure were calculated from an equation that describes two simultaneous first-order processes, and fit the data well. From such an equation, we

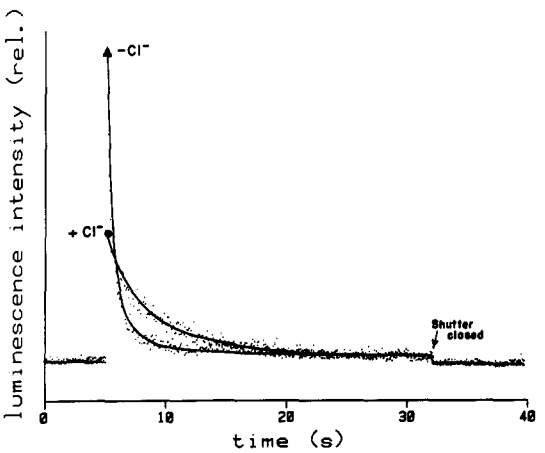

Fig. 6. Delayed light emission from Cl^--sufficient and deficient, DCMU-poisoned chloroplasts between 0.05 and 25 s after a constant illumination period. O, Cl^--sufficient thylakoids; △, Cl^--deficient thylakoids. Assay medium as in Fig. 5, except Chl concentration was 20 μg/ml. Assay conditions: $t = 0$, chloroplasts into assay medium and kept in the dark; $t = 1.5$ min, 1 μM DCMU added; $t = 2$ min, blue preilluminating actinic light on; $t = 2$ min 10 s, actinic light off. A shutter placed between the sample and photomultiplier tube was opened 50 ms after the actinic light was extinguished and closed again approx. 25 s later. The points in the figure are separated by 20 ms; the lines were drawn by hand.

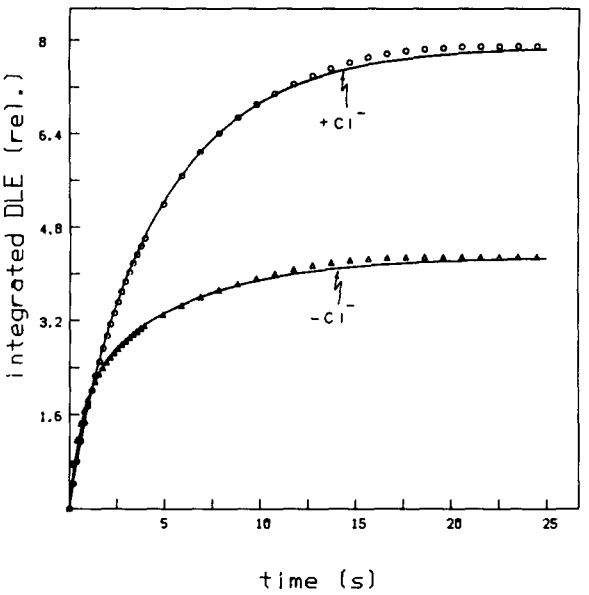

Fig. 7. Integrated delayed light emission from the luminescence decay curves shown in Fig. 6. O——O, Cl^--sufficient thylakoids; Δ——Δ, Cl^--deficient thylakoids. Points represent integrals of the data from Fig. 6 calculated by trapazoidal summation. The lines were calculated according to the biphasic kinetic equation given in Fig. 5, with the amplitudes of the slow and rapid phases now denoted by A_1 and A_2, respectively. The following parameters were used: $+Cl^-$: $A_1 = 7.11$, $k_1 = 0.2$ s^{-1}; $A_2 = 0.79$, $k = 1.0$ s^{-1}; $-Cl^-$: $A_1 = 2.36$, $k_1 = 0.18$ s^{-1}; $A_2 = 1.93$, $k_2 = 1.5$ s^{-1}.

determined that the rapid component of Q_a^- reoxidation was accelerated by Cl^- removal, whereas the slower component was retarded. When a simpler response to Cl^- removal was assumed, namely that a change occurred only in the slow phase of Q_a^- reoxidation, a good fit to the experimental data could not be obtained (dotted line).

To supplement the experiment just described, we also measured the delayed light emission from DCMU-poisoned, Cl^--depleted chloroplasts (Fig. 6). In this experiment, a shutter in front of the photomultiplier tube was opened 50 ms after the end of a 10-s preillumination period, and closed again after 25 s. It can be seen that the delayed fluorescence intensity was higher in the absence of Cl^- (first point indicated by the triangle), but that the two curves crossed at approx. 1 s. Qualitatively similar curves were obtained after a preillumination with a single flash. These results are opposite to those obtained by Muallem and Laine-Boszormenyi [16], who monitored delayed light emission from Cl^--depleted and -sufficient samples during a different time interval (between (0.15 and 3 min).

The time-course of the growth of integrated areas under the delayed light curves of Fig. 6 are shown in Fig. 7. These areas are proportional to the number of luminescence quanta emitted by the respective samples during the recorded time intervals. It can be seen that control samples emitted approximately twice as many photons between 0.05 and 25 s as did the corresponding Cl^--free chloroplasts. The Cl^--depleted thylakoids are further distinguished from the controls in this figure by an increase in the amplitude (from 10 to 45%) and rate constant (from 1.0 to 1.5 s^{-1}) of a rapid kinetic component of the area growth.

General discussion

The experiments in this paper were designed to address three questions concerning the structure and function of the oxidizing side of PS II in Cl^--depleted chloroplasts. First, we provide evidence which suggests that the inhibition of electron transport observed upon Cl^- removal is not due to the reversible release of functional Mn from the water-splitting complex, as is often the case with other inhibitory treatments of PS II. A similar conclusion was recently reached by Sandusky and Yocum [42] based on the power saturation

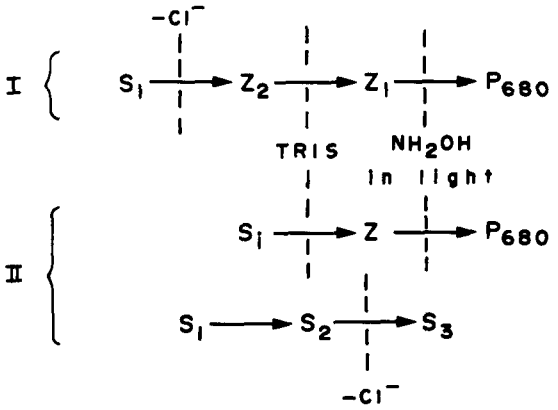

Fig. 8. Two possible arrangements of electron carriers on the oxidizing side of PS II. Dashed lines indicate the proposed sites of inhibition caused by the various treatments.

profile of EPR signal IIf in samples depleted of Cl^-.

A second major point addressed by our experiments concerns the functional location of the Cl^--dependent lesion in PS II. We used two techniques which monitor the lifetime of P-680$^+$ to count the number of electrons that could be rapidly extracted after a flash from the oxidizing side of PS II in Cl^--free chloroplasts. P-680$^+$ was detected as a fluorescence quencher in the experiments shown in Figs. 2 and 4, or as a substrate for delayed light emission in the experiments of Figs. 3 and 4c. Both types of experiments indicated that two electrons were available to reduce P-680$^+$ after Cl^- removal. This is in contrast to the effects of NH_2OH/light-treatment or Tris-washing, after which none, and one, electron(s) could be extracted by P-680$^+$, respectively (Fig. 2 and Refs. 20,22,31,32). We also discovered that the irreversible photoinactivation of Cl^--free chloroplasts apparently creates a new lesion in the electron transport chain at the same site as does NH_2OH incubation in the light.

Even though we are aware of the possibility that the reactions on the oxidizing side of PS II should be presented in a rather elaborate scheme [20], we shall discuss our data with the simple reaction schemes shown in Fig. 8. In terms of the two proposed schemes, treatment with NH_2OH in the light apparently blocks electron transport between P-680 and the secondary donor, Z or Z_1, and Tris-washing interrupts electron transfer between the secondary and tertiary donors, i.e., Z_2/Z_1 in scheme I or S-states/Z in scheme II. To account for the ability of Cl^--depleted chloroplasts to deliver two electrons to P-680, we have to assume that the Cl^--dependent lesion is between the water-splitting enzyme (S-states) and Z_2 in scheme I, or that Cl^- removal blocks the transition from S_2 to S_3 in scheme II.

An experiment designed to discriminate between these possibilities produced results which suggest, though not unambiguously, that NH_2OH delayed the detection of the fully Cl^--depleted state by two flashes. Since very low concentrations of NH_2OH were used which are unable to maintain the oxidizing side of PS II in a reduced state, a condition recognizable by lower, rather than higher delayed light emission [21], we feel that the most likely interpretation of our experiment is a direct action of NH_2OH on the S-states. Thus, we favor the second of the two schemes in Fig. 8.

Recently, Izawa et al. [17] concluded that the S_2 state in Cl^--free water-splitting complexes is reached through a two-step mechanism, namely, formation of S_2 in the Cl^--sufficient condition, followed by dissociation of Cl^-. If this is true, the Cl^--dissociation step would have to be completed within 100 ms to yield the results shown in Fig. 3. This seems unlikely, in view of the rather slow kinetics ($t_{1/2} > 10$ s) for the loss of Hill activity observed when chloroplasts are diluted into Cl^--free media [10]. These kinetics did not change dramatically when Cl^- release was determined as a function of flash number [43].

In choosing the second of the two schemes in Fig. 8, we cast serious doubt on the hypothesis [44–47] that the role of Cl^- is that of a counter ion to those stored positive charges on the water oxidase that have not been compensated by the release of a proton upon formation of S_2 [44,48–50]. In such a case, the S_2 state of Cl^--depleted chloroplasts should be highly unstable. Hence, an accumulation of two oxidizing equivalents, as seen in the experiments of Figs. 2 and 3, would be impossible, because only one electron would be available for P-680$^+$ reduction after Cl^- depletion, whereas the second would be consumed in the S_2 deactivation reaction. The same conclusion was recently reached by Velthuys [51], although by somewhat more indirect means.

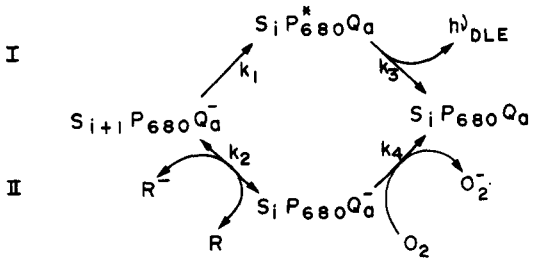

Fig. 9. Possible mechanisms for the regeneration of open PS II reaction centers after illumination of Cl^--sufficient and Cl^--deficient chloroplasts. For simplicity, the immediate electron donor to P-680, Z, is omitted. R^-, endogenous reductant of S_{1+1}; O_2^-; singlet oxygen radical. Deactivation pathway I leads to luminescence, whereas pathway II does not. Both k_1 and k_2 are proposed to be accelerated by Cl^- depletion.

The final point addressed by our experiments concerns the stability of the light-induced oxidants produced by a Cl^--free PS II. We monitored the rate of the back-reaction after a light flash or steady-state preillumination period, and found, surprisingly, that the results obtained by two different methods were, at first glance, contradictory. Whereas the luminescence experiments (Figs. 3 and 6) indicated that back-reactions producing excited P-680, the substrate for delayed light emission, were accelerated in the absence of Cl^-, the prompt fluorescence data obtained under similar conditions (Fig. 5) suggested a stabilization of the reduced secondary acceptor, Q_a^-.

This apparent paradox can be resolved by considering the reactions proposed in the scheme of Fig. 9. In it, an endogenous reductant, R^-, is assumed to compete with Q_a^- for some of the oxidizing equivalents on the water-splitting enzyme, producing S_iP-680Q_a^-. Electrons are then only slowly extracted from Q_a^- by an oxidant, perhaps oxygen. The reduction of the formed oxidant, R, may be part of the process of photoinhibition of Cl^--free chloroplasts.

In the presence of Cl^-, pathway II is assumed to be largely inoperative, but in Cl^--depleted thylakoids, R^- becomes an efficient reductant of the S-states. The regeneration of open PS II reaction centers would therefore become increasingly slower as more oxidizing equivalents are lost to R^-. In pathway I, k_1 is thought to be increased as the S-states are destabilized by a lack of functional Cl^-, resulting in faster production of P-680$^+$, and thus, increased delayed fluorescence intensity.

This model is successful in qualitatively predicting a number of our observations. First, if the rapid and slow kinetic phases of Q_a^- reoxidation observed in the fluorescence experiment of Fig. 5 reflect pathways I and II described above, then the faster component should be accelerated by Cl^- removal, while the slower should be decelerated. Secondly, because k_1 is thought to be increased in the absence of Cl^-, the intensity of delayed light emitted from these samples should start out higher, but since oxidants are drained off by pathway II, luminescence should end earlier. Finally, the total number of quanta emitted as delayed fluorescence should be smaller in Cl^--free samples, while the kinetics of the area growth should be accelerated. All of these results were observed in our experiments, although conclusions based on Fig. 7 are weakened by the fact that we were not able to quantitate the contribution of the delayed light emitted at $t < 50$ ms to the total integrated light sum.

Our conclusion that the state S_{i+1}P-680Q_a^- deactivates more quickly in the absence of Cl^- contradicts Muallem and his co-workers' [14,16,17,19] contention that Cl^- removal stabilizes the higher S-states. Their interpretation was rather convincingly supported by an observed lowering of the luminescence intensity caused by Cl^- depletion. Since we never saw such an effect, we wonder whether this discrepancy is in part caused by the different time domains covered by our respective experiments.

This difference in experimental protocol cannot account for their findings that Cl^- addition to Cl^--free chloroplasts caused an immediate enhancement of delayed light emission [14,16], and that the concentrations of S_2 and S_3 in dark-adapted, Cl^--depleted samples measured with a rate electrode appeared to be much higher than usual [14]. One explanation could be that Cl^- binding to the water-splitting complex facilitates a dark transition from the S_1 to S_2 state. This could occur if the reaction denoted by k_2 in Fig. 9 is in fact reversible, and that the reverse step (though k_{-2}) is inhibited by Cl^- removal. This requires the assumption that the midpoint potential of either the R^-/R or S_1/S_2 couples is grossly changed upon Cl^- binding and removal.

After completion of this work, it came to our attention that experiments very similar to those depicted in Fig. 2 involving Cl^--deficient chloroplasts had been independently carried out in Dr. Croft's laboratory. Their results are published in an accompanying paper [51], and to the extent that our experiments overlap, our respective data and interpretations are in excellent agreement.

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