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EPR evidence for a modified S-state transition in chloride-depleted Photosystem II

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The role of chloride on the S-state transition in spinach Photosystem II (PS II) particles was investigated by EPR spectroscopy at low temperature and the following results were obtained. (1) After excitation by continuous light at 200 K, chloride-depleted particles did not show the EPR multiline signal associated with the S_2 state, but only showed the broad signal at $g = 4.1$. The S_2 multiline signal was completely restored upon chloride repletion. (2) In the absence of chloride the S_2 multiline signal was not induced by a single flash excitation at 0°C. However, upon addition of chloride after the flash the signal was developed in darkness. (3) The amplitude of the multiline S_2 signal thus developed upon chloride addition after flash illumination did not show oscillations dependent upon flash number. These results indicate that the O_2-evolving complex in chloride-depleted PS II membranes is able to store at least one oxidizing equivalent, a modified S_2 state, which does not give rise to the multiline signal. Addition of chloride converts this oxidizing equivalent to the normal S_2 state which gives rise to the multiline signal. The modified S_2 state is more stable than the normal S_2 state, showing decay kinetics about 20-times slower than those of the normal S_2 state, and the formation of higher S states is blocked.

Introduction

It has been well established that chloride is an indispensable cofactor involved in photosynthetic O_2 evolution [1]. Earlier work suggested that the functional site of chloride is the O_2-evolving complex, since chloride depletion does not inhibit electron donation from artificial electron donors to PS II [2]. Muallem et al. [3] provided the first evidence that the O_2-evolving system could accumulate oxidizing equivalents in the absence of chloride, although O_2 evolution was lost. Their results suggested furthermore that these oxidizing equivalents were unusually stable as compared with the normal S_2 and S_3 states. Higher S-state formation in chloride-depleted conditions was analyzed quantitatively by Theg et al. [4] and Itoh et al. [5]. By measuring the yield change of chlorophyll fluorescence due to reduction of P-680$^+$, they counted the number of electrons originating from the donor side of PS II which were available to reduce the photooxidized P-680, the PS-II reaction center chlorophyll. It was concluded that the transition from S_1 to S_2 proceeded normally but

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Abbreviations: PS, Photosystem; Chl, chlorophyll; Mes, 4-morpholineethanesulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Z, the secondary donor of Photosystem II; Q_B, the secondary quinone acceptor of Photosystem II; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

that further transitions beyond S_2 were blocked in the absence of chloride; in contrast to the earlier work [3], the chloride-free S_2 state deactivated with unusually rapid kinetics [4].

More recently, Homann and his co-workers [6,7] have used thermoluminescence measurements to characterize the events during oxidant accumulation in chloride-free PS II membranes. They found emission bands possibly corresponding to S_2 and S_3 in the absence of chloride, but the shape and the emission temperature of the bands were greatly modified. It was inferred that in the absence of chloride, S_2 and S_3 states with unusually high stabilities could be formed, but that S_4 formation was blocked. Inhibition of the S_3 to S_4 transition when nitrate was substituted for chloride has also been suggested by Sinclair [8].

Although these reports are not always consistent with regard to the inhibitory site of chloride depletion nor the properties of the chloride free S state(s), it can be concluded that one or more oxidants can be accumulated in the O_2-evolving complex when inhibited by chloride depletion [3–8].

Direct detection of the S_2 state is now possible by EPR spectroscopy. The light-induced multiline signal observed at low temperature arises from S_2 [9–12]. This EPR signal has been proposed to arise from interactions between two or four manganese atoms [9,13–15]. Studies of the EPR multiline signal should be a useful tool to understand the effect of chloride depletion on the S-state transition in terms of the oxidation state of the manganese atoms in the cluster. In the brief studies reported so far, conflicting results have been obtained. Brudvig et al. [16] reported gross modifications of the S_2 multiline signal, while Franzen et al. [17] found no effect upon chloride depletion. In the present study, we have investigated the effect of chloride depletion and repletion on S-state formation in the O_2-evolving complex of PS II particles by EPR spectroscopy at low temperature. We show that the multiline signal is not induced by illumination of the chloride-depleted particles, but that the signal is developed upon addition of chloride after illumination. It is concluded that an S_2 state with modified properties is formed in the absence of chloride, and that the further transition beyond the modified S_2 state cannot occur.

Materials and Methods

Triton X-100 PS II particles capable of O_2 evolution [18] were prepared as previously described [19], and stored in liquid N_2. The particles were suspended in 0.4 M sucrose/20 mM NaCl/2 mM EDTA · Na_2/40 mM Mes-NaOH (pH 6.5), and centrifuged at $35\,000 \times g$ for 10 min. The pellet was washed twice with 0.4 M sucrose/2 mM NaCl/4 mM Mes-NaOH (pH 6.5) and centrifuged at $35\,000 \times g$ for 10 min. The following treatments were carried out under dim green safe light.

In PS II particles, a rapid depletion of chloride can be successfully attained by a brief alkaline treatment or by a substitution by sulfate [6,7]. The latter was the easier procedure and was more reproducible [7], although for complete depletion the treatment had to be performed at pH 7.5 [20]. Thus, for chloride depletion, the particles were suspended in 0.4 M sucrose/50 mM Na_2SO_4/40 mM Hepes-NaOH (pH 7.5) at a chlorophyll concentration of 0.4 mg Chl/ml, incubated for 10 min in the dark and then centrifuged at $35\,000 \times g$ for 10 min. The resulting pellet was resuspended in the same medium and was used as chloride-depleted particles. For rapid chloride repletion, NaCl was added to the chloride-depleted particles to a final concentration of 100 mM either before or after the particles were placed in the EPR tube (see legends to figures). The chloride-sufficient control particles were washed once in 0.4 M sucrose/50 mM NaCl/40 mM Hepes-NaOH (pH 7.5) buffer and were resuspended in the same medium.

EPR samples in calibrated quartz tubes were further incubated in darkness for 10 min at 20°C. The samples were illuminated either with continuous light for 4 min at 200 K in an ethanol/solid CO_2 bath or with a series of flashes provided from a Nd-YAG laser (15 ns, 100 mJ, 530 nm) at 0°C at an interval of 1 s as described [12]. The light intensity of the laser flash was confirmed to saturate the PS II reaction based on the oscillation pattern of the multiline signal (Fig. 4). The flash-illuminated samples were quickly cooled to 200 K and then stored at 77 K. When indicated, 100 mM 2,5-dimethylquinone dissolved in dimethyl sulfoxide was added to give a final concentration of 1

mM as an electron acceptor. The samples contained no glycerol, since it has been shown to modify the characteristics of the multiline signal [12] and of the $g = 4.1$ signal [21].

EPR spectra were recorded at liquid helium temperature with a Bruker ER-200t-X-band EPR spectrometer equipped with an Oxford Instruments cryostat as described [12]. A Tracor-Northern 1710 apparatus was used for averaging and subtraction of spectra.

O_2 evolution was measured with a Clark-type oxygen electrode with 2,5-dimethylquinone (2 mM) as an electron acceptor at 25°C in 400 mM sucrose/40 mM Hepes-NaOH (pH 7.5) supplemented with salts as described in Ref. 19.

Results

Fig. 1A shows the EPR spectra of PS II particles in chloride-sufficient (a) and chloride-depleted (b) conditions. After continuous illumination for 4 min at 200 K, which accumulates the S_2 state, a large multiline signal was induced between 2500 and 4000 G, as reported previously [11], in the chloride-sufficient control particles. In addition, the broad structureless signal at $g = 4.1$ previously reported under these conditions [12] is also induced. The sharp signal at $g = 4.3$ which is present in the dark is due to rhombic ferric iron and is present as a background signal in these preparations. In the chloride-depleted particles, however, almost no multiline signal was photoinduced, but the broad signal at $g = 4.1$ was still largely photoinduced. In chloride-depleted samples, a small $Mn^{2+}(H_2O)_6$ signal is present in the dark. Thawing and refreezing increased the amplitude of this signal (data not shown). This reflects an unusual instability of the manganese in the chloride-depleted preparation.

The features of the light-induced EPR signals are better seen in the difference spectra (light minus dark) as shown in Fig. 1B. It is clearly shown that chloride depletion completely inhibited the formation of the multiline signal, while the signal at $g = 4.1$ observed between 1200 and 2200 G was much less affected by the depletion. The photoinduced signal at $g = 1.9$, which has been attributed to a reduced form of the primary quinone acceptor of PS II [22], was still evident

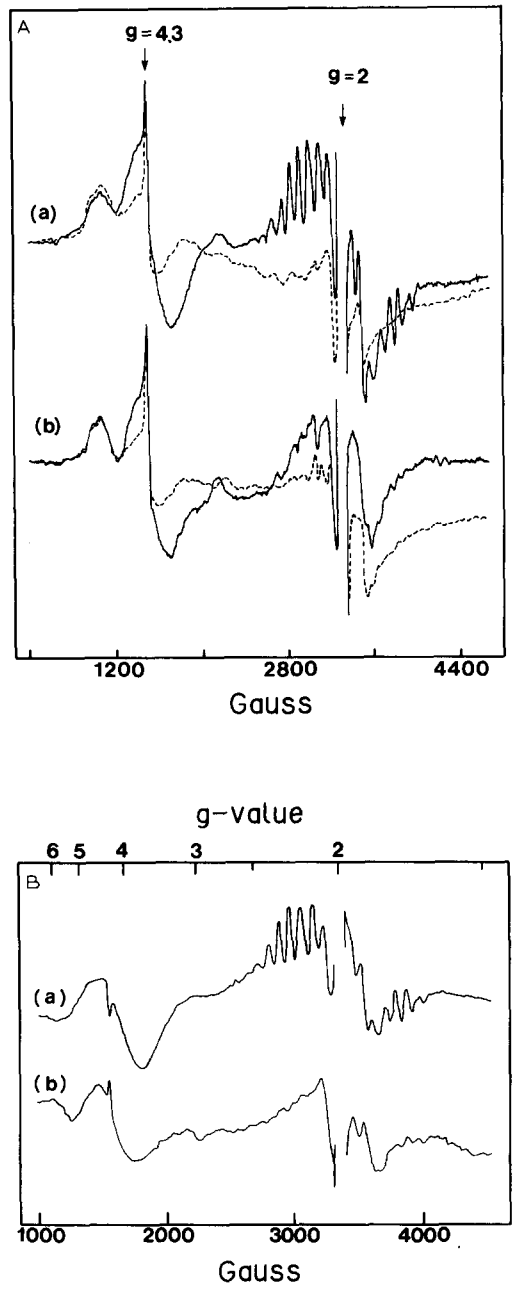

Fig. 1. Effect of chloride depletion on the EPR signals induced by continuous illumination for 4 min at 200 K. A, spectra of dark-adapted (broken line) and illuminated (solid line) PS II particles. (a) control; (b) chloride-depleted. B, difference spectra (after illumination minus dark) of (a) control PS II particles, and (b) chloride-depleted PS II particles. Instrumental settings: temperature, 8 K; microwave power, 2 mW; microwave frequency, 9.43 GHz; modulation amplitude. 32 G. Average of two scans in B. Chlorophyll concentration was 6.1 and 5.2 mg Chl/ml for (a) and (b), respectively.

with an approximately similar amplitude after chloride depletion, thus showing that photochemistry occurs to approximately the same extent in both samples. The $g = 1.9$ signal present in the dark spectra is largely due to the Rieske iron sulfur centre which is present as a contaminant in the PS II preparation used here.

As shown in Table I, when chloride was added to the depleted particles, the ability to form the multiline signal by illumination at 200 K was restored to more than 80% of the control concurrently with 70% restoration of O_2 evolution. However, the EPR signal at $g = 4.1$ was nearly independent of the depletion and repletion of chloride. The hyperfine structure of the multiline signal restored by chloride repletion was identical to that of the control particles (data not shown).

Fig. 2 shows the effect of depletion and rapid repletion of chloride on the multiline EPR signal induced by a single flash at 0°C. In this experiment, chloride was re-added to the depleted particles by one of the following two different procedures: firstly, chloride was added to the sample in the EPR tube just before flash excitation (trace (b)); secondly, chloride was added just after flash excitation followed by a dark mixing period at 0°C for 40 s (trace (c)). As shown by trace (a), the chloride depletion inhibited the formation of the multiline signal by a flash, consistent with the result obtained by continuous illumination at 200 K (Fig. 1). It was, however, possible to develop the multiline signal by the addition of chloride after the flash. The amplitude of the multiline signal generated by the chloride addition after the flash amounted to 60–80% of that of the

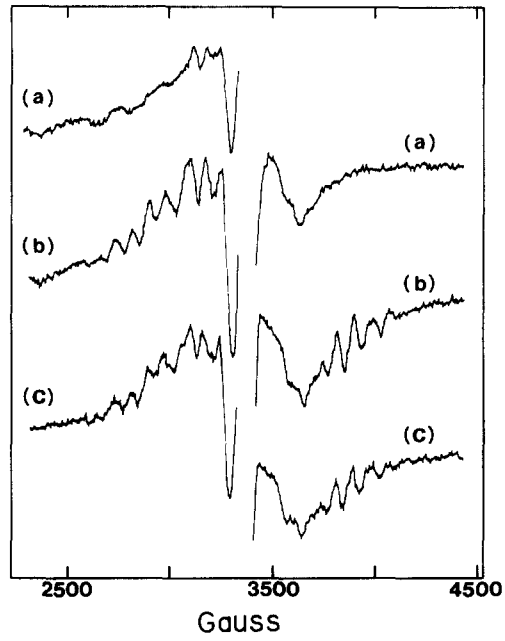

Fig. 2. Effect of chloride depletion and chloride repletion on the multiline EPR signal induced by a single flash at 0°C. (a) chloride-depleted PS II particles; (b) chloride-repleted PS II particles to which chloride was added before flash illumination; (c) chloride quick-repleted PS II particles to which chloride was added immeidately after flash illumination followed by dark mixing for 40 s at 0°C before being cooled rapidly to 77 K. The instrument settings were the same as in Fig. 1. Chlorophyll concentration was 3.0 mg Chl/ml.

TABLE I

EFFECTS OF DEPLETION AND REPLETION OF CHLORIDE ON EPR SIGNALS AND O_2 EVOLUTION

The control oxygen evolution rate was 530 μmol O_2/mg Chl per h.

Condition	Multiline signal (relative amplitude)	$g = 4.1$ signal (relative amplitude)	O_2 evolution (%)
Control	100	100	100
Cl^--depleted	< 10	80	4
Cl^--depleted and reconstituted	82	83	65

chloride-repleted particles prior to the flash. The slightly lower amplitude of the dark-developed multiline signal is at least partially due to some deactivation of S_2 during the dark mixing period of 40 s (see Fig. 4). This result indicates that an oxidizing equivalent can be stored on the O_2-evolving complex in the absence of chloride, even though the capacity to evolve O_2 is almost completely lost.

We measured the stability of this oxidant in comparison with that of the normal S_2 state. The chloride-depleted particles were illuminated with a single flash at 20°C, incubated at this temperature for various dark times, cooled to 0°C, and chloride was added. The sample was then frozen and the amplitude of the multiline signal was plotted against the dark time. As shown in Fig. 3A, both the multiline signals induced by one flash in the control sample (a) and in the chloride-depleted

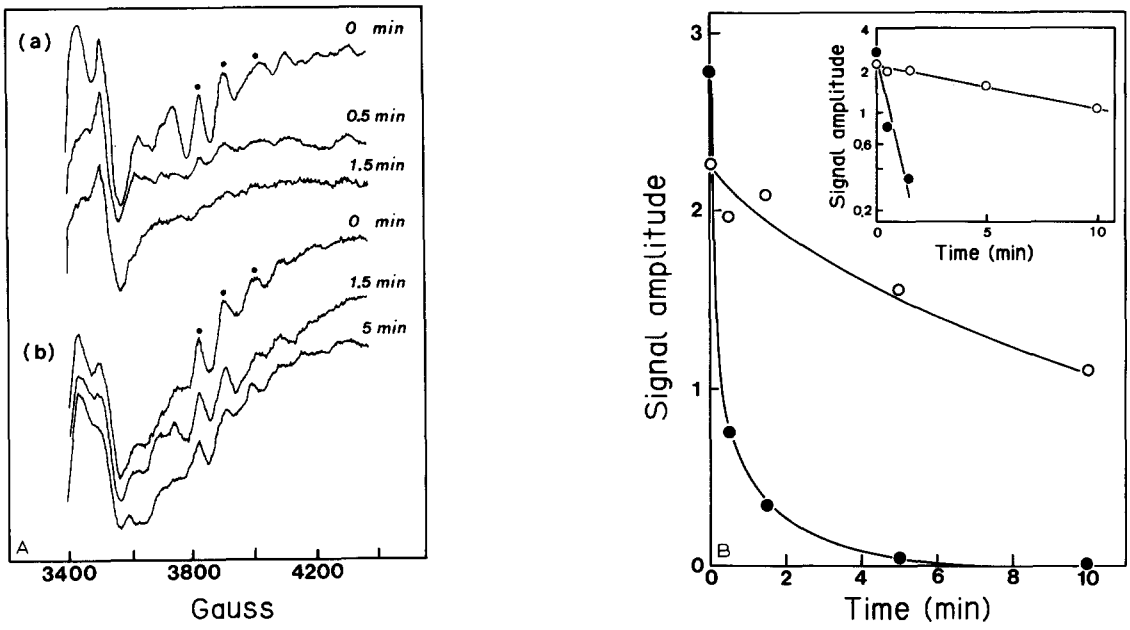

Fig. 3. Deactivation of the modified S_2 state measured as the amplitude of the multiline EPR signal formed on quick repletion of chloride after flash excitation. A, multiline signal decay in (a) control PS II particles measured during dark incubation at 20°C, and (b) the multiline signal developed in chloride-depleted PS II particles by quick addition of chloride at 0°C. B, decay course of the multiline signal in control (●) and in chloride depleted (○) PS II particles. The amplitude of the multiline signal was estimated from the height of the three highfield peaks marked (●) and plotted against the incubation time. Inset is a semilogarithmic plot. Instrument settings: temperature, 20 K; microwave power, 50 mW; microwave frequency, 9.42 GHz; modulation amplitude, 32 G. Chlorophyll concentration was 3.0 mg Chl/ml.

sample (b) decayed during the dark incubation time. However, there was a marked difference in the decay kinetics (Fig. 3B): the normal S_2 state showed a monophasic decay with an approximate half-time of 0.5 min, whereas the modified S_2 state decayed monophasically with a half-time of 10 min. The observed decay kinetics of the normal S_2 state is in a similar time range to that reported for native PS II particles by other investigators [11,12,19]. A 20-fold slower deactivation of the modified S_2 state indicates that the oxidized equivalent formed in the absence of chloride is much more stable than S_2.

Since the multiline signal arises from S_2 [9–12], the amplitude of the signal shows an oscillatory behavior depending on the flash number, with maxima on the first and fifth flashes and a minimum on the fourth flash [9,12,17]. As shown in Fig. 4, our control particles showed a flash number-dependent change in the amplitude of the multiline signal, with a maximum on the first flash followed by a sharp decline on the second and

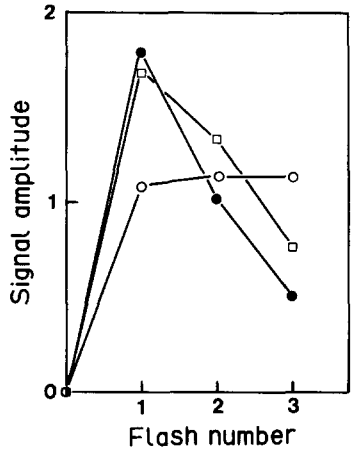

Fig. 4. Amplitude changes of the multiline signal after a series of flashes at 0°C. Control PS II particles (●); chloride-repleted PS II particles (□), to which chloride was added to depleted particles just before being placed in EPR tubes and before flash illumination; chloride quickly repleted PS II particles (○), to which chloride was added to depleted particles immediately after a series of flashes. 2,5-Dimethylquinone was added to give a final concentration of 1 mM as an electron acceptor. Instrument settings and the estimation of signal amplitude were the same as in Fig. 3. Chlorophyll concentration was 3.0 mg Chl/ml.

third flashes, in agreement with previously reported patterns [9,12,17]. In contrast, the amplitude of the multiline signal developed in chloride-depleted particles by the addition of chloride after a series of flashes did not show such flash number-dependent variations but showed a constant amplitude after the second flash. When chloride was repleted prior to the flash, the oscillatory behavior of the signal amplitude was restored to nearly the same extent as in the control particles. This result indicates that no further oxidizing equivalents are stably stored on the oxygen-evolving complex after the first flash.

Discussion

The results presented in this study demonstrate that in chloride-depleted PS II particles neither a flash excitation at room temperature nor continuous illumination at 200 K induces the multiline EPR signal arising from the S_2 state. This observation is in contradiction with the report of Franzen et al. [17] who found that chloride depletion did not affect the formation of the multiline signal. The origin of this discrepancy is not understood but could lie in differences in the procedure used for chloride depletion. Nevertheless, the loss of the multiline signal in this work is due to depletion of chloride, since the repletion of chloride restores the ability to form the multiline signal. The multiline signal can also be developed by addition of chloride after the flash excitation of the chloride-depleted particles. This shows that an oxidizing equivalent can be stored on the donor side of PS II in chloride-depleted particles. The question arises, on which component is this oxidizing equivalent localized? Since no multiline signal is observed, it is clearly not a normal S_2 state. It is possible that the positive charge is located on a component other than the manganese cluster of the O_2-evolving enzyme. However, no changes in cytochrome *b*-559 or Signal II were detected nor were any other EPR signals detected which could be attributed to the charge storage state (data not shown).

Some information on the whereabouts of the positive charge comes from observations of the $g = 4.1$ signal. This signal, although originally proposed to be due to a component operating as a carrier between the reaction centre and the O_2-evolving complex [12,23], has recently been proposed to arise from the S_2 state [21,24]. Evidence has been obtained indicating that the $g = 4.1$ signal arises from S_2 in a subpopulation ($< 20\%$) of the centres while the majority of the centres gives rise to the multiline signal [21]. In the current work, it is shown that the $g = 4.1$ signal is unaffected by chloride depletion. This could mean either that these centres are not susceptible to chloride depletion (this is unlikely since O_2 evolution is almost completely inhibited) or that these centres are affected by chloride removal but that the effect is not manifest as a change in the EPR spectrum. If the latter explanation is correct, this would be an indication that a true modified S_2 state (i.e., a positive charge stored on the manganese cluster) was formed in chloride-depleted centres.

The existence of the S_2 state in a form which does not give rise to the multiline signal has already been suggested [17,25] to explain the absence of the multiline signal under conditions in which S_2 is thought to be present, i.e., when the 24 kDa protein was removed [17], and in similar samples in which oxygen evolution was partially reconstituted by readdition of the 24 kDa protein [26] (for a review see Ref. 25). It is reasonable that the manganese atoms in the chloride-free O_2-evolving centre are capable of storing an oxidized equivalent similar to S_2 but which is modified so that it does not give rise to the EPR multiline signal as a result of a chloride depletion-induced change in the interaction between the manganese atoms in the O_2-evolving centre.

It has been suggested that the role of the 24 kDa and 16 kDa proteins is to increase the affinity of the O_2-evolving complex for chloride [1]. In this respect, Toyoshima et al. [27] observed that in the absence of these two proteins the multiline signal could be formed when chloride was present but not when chloride was left out of the buffer. Although this was originally interpreted as an inhibition of S_2 formation which could be restored by readdition of the proteins or of chloride, it seems likely that these results can be reinterpreted in terms of the chloride-depletion effect characterized here. Accordingly it would be predicted that a modified S_2 state, like that described here, was in

fact present in the experiments of Toyoshima et al. [27] when no multiline signal was observed.

The multiline signal has been attributed to a mixed-valence manganese cluster involving two or four manganese atoms [9,10,13–15]. It has been postulated that chloride could act as a ligand to the manganese atoms of the oxygen-evolving complex where it could play a variety of different roles including stabilization of higher S states [28], facilitation of electron transfer within and out of the cluster [29], etc. (see Ref. 1 for a review of other data and hypotheses). If chloride were directly liganded to manganese its removal would certainly be expected to result in marked changes in the multiline signal. However, minor configurational changes could sufficiently perturb the manganese cluster to produce a system in which the multiline signal would not be present (see Ref. 14). Indeed, the fact that the $g = 4.1$ signal is unchanged by chloride depletion might indicate that the changes in the manganese cluster are rather slight. A role for chloride involving protonation of groups on the polypeptides which form the functional cavity for the manganese cluster has also been suggested [6,7,30]. Chloride depletion resulting in configurational changes in these polypeptides could equally well result in the loss of the multiline signal, particularly since it has been suggested that the manganese cluster may bridge two different polypeptides [21].

The modified S_2 state shows other properties which are distinctly different from those of the normal S_2 state. Firstly, the decay kinetics of the modified S_2 state ($t_{1/2} = 10$ min) was about 20-times as slow as that of normal S_2 ($t_{1/2} = 30$ s) (see Fig. 3). This remarkably slow deactivation of the modified S_2 state agrees with the result of Muallem et al. [3] that a long-lived S_2 state was present in 40% of the centres in the chloride-depleted chloroplasts after 6 min dark adaptation. Similarly, Homann et al. [7] reported that chloride depletion alters the thermoluminescence B-band to show a very high emission temperature, which in turn indicates the generation of a highly stable charge pair in chloride-depleted conditions. Theoretical analysis of the modified features of this thermoluminescence band revealed that the redox potential of the modified S_2 is significantly lower than that of normal S_2 [7]. The abnormally high emission temperature of the B-band in chloride-depleted sample can be quickly reversed to normal temperature on addition of chloride after flash illumination, and the charge pair after the chloride addition shows a decay course typical of the normal S_2 [7]. This indicates that chloride converts the modified S_2 to normal S_2, in agreement with the EPR results in this paper.

This result, however, contradicts the conclusion of Theg et al. [4] who proposed that the S_2 state formed in chloride-depleted conditions was less stable than normal S_2. This interpretation was based on apparently contradictory data obtained by measurements of fluorescence and luminescence. It seems likely, however, that the data can be reinterpreted in terms of a model in which the modified S_2 state is more stable than the normal S_2 state as concluded from the EPR work reported here. A longer-lived S_2 state would give rise to a longer-lived Q_A^- in DCMU-treated material, since it normally decays by recombination with S_2. This is consistent with the higher emission temperature of the thermoluminescence of $S_2Q_A^-$ recombination in chloride-depleted material [7] and would explain the increased slow phase of fluorescence quenching and also the decrease in the seconds time-scale luminescence attributed to $S_2Q_A^-$ recombination (see Ref. 31). Instead, very long-lived luminescence would be expected due to Q_A^- recombination with the modified S_2 as was observed earlier [3]. The increase in short-lived luminescence and the speeding up of the fast-phase fluorescence quenching [4] could be due to charge recombination in a fraction of centres in which the positive charge did not arrive on the modified S_2 state, due perhaps to photodamage of the chloride-depleted material.

Secondly, the failure to observe a decrease in the amplitude of the dark-developed multiline signal by illumination with more than two flashes implies that the further transition beyond the modified S_2 state is blocked in chloride-depleted conditions (see Fig. 4). This result is consistent with the P-680$^+$ re-reduction measurements, using chlorophyll fluorescence as probe, by Theg et al. [4] and Itoh et al. [5]. They observed that in the absence of chloride photo-oxidized P-680$^+$ was rapidly reduced after the first and second flashes but not after subsequent flashes, and concluded

that the transition from S_2 to S_3 was blocked by chloride depletion while the transition from S_1 to S_2 proceeds normally.

This result disagrees with the recent results obtained by thermoluminescence measurements which suggested that the modified S_2 can proceed to a modified S_3 [6,7]. This conclusion is mainly based on the observation that in chloride-depleted particles, the flash yield of thermoluminescence remarkably decreased at the second flash but kept a constant level after the third flash. The decrease in the emission yield at the second flash has been interpreted as indicating an advancement from $S_2O_B^-$ to S_3Q_B via $S_3Q_B^{2-}$ after the second flash, since the loss of electron on Q_B leads to a decrease in thermoluminescence arising from $S_2Q_B^-$ and $S_3Q_B^-$ recombinations [32]. However, a similar loss of the electron on Q_B^- would also be predicted if the second flash generated the $S_2Z^+Q_B$ state (Z: the secondary donor of PS II). This view agrees with the report that Z^+ is unusually stable in chloride-depleted thylakoids [5]. Thus the thermoluminescence data [6,7] can be reconciled with the conclusion made here and earlier [4,5] that S_2 is the highest S state formed in chloride-depleted material. It must be taken into account, however, that the effects of the various procedures generally referred to as 'chloride depletion' differ from each other in their details: i.e., nitrate replacement only slows down the S-state turnover rate [8], while sulfate replacement completely inhibits O_2 evolution [33].

It has been argued that the modified S_2 state formed under chloride-depleted conditions is formed by a chloride-independent S_1 to S_2 transition followed by the loss of chloride from S_2 to form the modified state [34]. The EPR work argues against this model, since the modified S_2 state is formed at 200 K in the frozen state. From the results reported here it seems more likely that the S_1 to normal S_2 transition does require chloride. The fact that normal S_2 can be formed in the frozen state in chloride-sufficient conditions can be explained by chloride already being close to its functional site prior to S_2 formation. The data do not provide information on whether S_1 is modified by chloride depletion.

Recent NMR work using PS II from a halophyte has shown that chloride is bound only at the S_1 to S_2 and S_2 to S_3 steps [35]. From the data reported here it is proposed that these chloride-binding steps have the following effects: (1) in the presence of chloride the manganese cluster attains a conformation in which the multiline signal is observable from the S_2 state; (2) chloride destabilizes the S_2 state and makes it more reactive, probably due to an increased redox potential relative to that in the absence of chloride; (3) chloride allows the S_2 state to advance to S_3.

During revision of this paper, a paper by Damoder et al. [36] was published. They also observed a reversible partial loss of the S_2 multiline signal in chloride-depleted PS II membranes, but attributed the loss to an incomplete turnover of S state in the absence of chloride due to (1) disconnection of the Mn complex from the reaction centre, and/or to (2) deactivation of a part of S_1 centres having reduced Q_A to form S_0 centres. Both their observation and their interpretation completely disagree with ours: they consider that the EPR multiline active S_2 can be formed in the absence of chloride, if only electron abstraction from the Mn complex is attained by repeated illumination, while we consider that illumination of chloride-depleted samples results in formation of an EPR-silent abnormal S_2, and the abnormal S_2 can be converted to the EPR-active normal S_2 in darkness if chloride is added within the lifetime of the abnormal S_2. Our idea that EPR-active S_2 will not appear unless chloride is added is totally inconsistent with their idea, and it appears impossible at present to reconcile the observations by the two groups. The most likely explanation would be to ascribe the inconsistencies as being due to differences in the methods of chloride depletion and in sample properties and treatment.

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