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Storage of abnormal oxidants 'Σ_1', 'Σ_2' and 'Σ_3' in photosynthetic water oxidases inhibited by Cl^--removal

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The Cl^- requirement of photosynthetic O_2 evolution was studied by thermoluminescence measurements with purified Photosystem II-containing membrane particles from chloroplast thylakoids. When Cl anions had been removed from the particles either by an alkaline shock in a Cl^--free medium, or by treatment with SO_4^{2-}, the pattern of the thermoluminescence emission after illumination with increasing numbers of flashes suggested that the oxidant storage in the water oxidase could only proceed up to the final step. The final step itself, i.e., the advance to the water-oxidizing S_4 state, apparently was blocked. An upward shift of the emission temperatures of the thermoluminescence bands was seen both in the absence and in the presence of 3-(3′,4′-dichlorophenyl)-1,1-dimethyl urea, indicating that the stored oxidants had redox properties different from those of normal, Cl^--sufficient preparations. These properties were readily interconvertible by addition or removal of Cl^-. We postulate that in Cl^--deficient water oxidases abnormal S_1, S_2 and S_3 states, symbolized as Σ_1, Σ_2 and Σ_3, respectively, are formed which are in a Cl^--dependent equilibrium with the corresponding normal S states. An oxidation of Σ_3 to a Σ_4 state is not possible. It is proposed that Cl^- controls the oxidation potential of the stored oxidants by regulating events associated with the binding and/or oxidative modification of water molecules at the water oxidase.

Introduction

Photosynthetic oxygen evolution becomes reversibly inhibited when thylakoid membrane preparations are depleted of Cl anions [1,2]. Since the discovery of the 'Cl^- effect' 40 years ago [3] it has become evident that the anion is required specifically, and perhaps exclusively, for the function of the photosynthetic water oxidase [4]. In the presently accepted view, this enzyme, or enzyme complex, is endowed at its active site with a cluster of presumably four Mn for the accumulation of the four oxidizing charges needed to produce one O_2 molecule from water. The states of oxidation are generally referred to as S_0 through S_4, the latter reacting spontaneously with water to produce O_2 and regenerate S_0 [5,6].

Izawa and his coworkers [7,8] were the first to show that, in spite of their inability to evolve O_2, illuminated Cl^--deficient thylakoids stored at least one oxidizing charge in addition to the one already present in the dark-adapted state. Since the

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Abbreviations: CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; Chl, chlorophyll; DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethyl urea; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, 4-morpholineethanesulfonic acid; Mops, 4-morpholinepropanesulfonic acid; PS II, Photosystem II.

primary donor Z between the reaction center P-680 and the S complex usually becomes only transiently oxidized, it was concluded that in the absence of Cl^- the S_2 and perhaps even the S_3 state can still be created. The authors provided evidence, furthermore, for an unusual stability of the light-generated oxidants in Cl^--deficient thylakoids. In contrast, the decay kinetics of chlorophyll luminescence reported by Theg et al. [9] suggested for Cl^--deficient thylakoids an unusually rapid reduction of S_2 by the reduced acceptor Q_A^-.

Recently, a more quantitative analysis of the charge accumulation process in the absence of Cl^- was performed independently by two groups of investigators [9,10]. Using chlorophyll fluorescence as probe, they found that the reaction center P-680 of a Cl^--depleted PS II, when photooxidized by light flashes, could receive for rereduction with 50 μs two electrons in succession. Thus it appeared that in the absence of Cl^- at least two oxidizing equivalents can be stored on the donor side of P-680. This result was interpreted as an unimpaired advance from P-680ZS_1 of the dark-adapted preparation to P-680Z^+S_2. However, a further reaction step to P-680ZS_3 was suggested by Sinclair [11] who concluded from O_2 measurements that a displacement of the functional Cl^- by SO_4^{2-} caused a block of the charge-accumulating sequence at the last step $S_3 \rightarrow S_4$.

In order to improve our understanding of the effects of Cl^- removal on the oxidant accumulating events in PS II, we took advantage of recent advances in the phenomenological and theoretical analysis of thermoluminescence [12–15]. This is the light emission caused by thermally induced recombination reactions between stored oxidants in the water oxidase and the reduced acceptors Q_A^- or Q_B^-.

As described already in a preliminary report [16], our data have provided evidence that S_2 and S_3 states with unique properties are formed in Cl^--deficient thylakoids, but that an advance from S_3 to the water-oxidizing S_4 state is not possible. In this article, we further substantiate those results, and show that the abnormal S states are in a Cl^--dependent equilibrium with the normally encountered ones. These findings suggest that Cl^- has a decisive influence on the oxidant storage in the Mn cluster of the water oxidase.

Materials and Methods

All experiments reported here were performed with membrane particles containing a purified PS II. They were prepared by Triton X-100 treatment of spinach (*Spinacia oleracea*) thylakoids as described elsewhere [17,18] and usually stored at 77 K in 20 mM Na-Mes (pH 6.5)/400 mM sucrose/ 10 mM NaCl/30% ethyleneglycol. Before use, they were washed several times with the same medium lacking the ethyleneglycol and NaCl. Occasionally, 15 mM Na_2SO_4 was included in the wash medium to guarantee a more thorough removal of adventitiously bound Cl^-. However, this modification had no discernible advantageous effect. The washed particles were suspended at 2–3 mg Chl/ ml in 400 mM sucrose buffered with 5 mM Mes (pH 6.3), and dark-adapted overnight on ice. In the experiment of Fig. 5, 3 mM NaCl was present in the dark-adapted suspension of thylakoid particles.

For the measurements, appropriately diluted (200–300 μg Chl/ml) suspensions, were kept on ice in media containing 400 mM sucrose, plus buffer and salts as indicated. Even when washed and stored under Cl^--free conditions, however, the water-oxidizing complexes of the preparations usually do not become depleted of functional Cl^- [18]. As special depletion procedures we used either a 5–10 s exposure of diluted suspensions to a Cl^--free medium of pH 9.6 [18], or we removed Cl^- by incubating diluted suspensions at pH 7.5 with 50–100 mM Na_2SO_4 for at least 5 min [10,11,19]. The depleted samples, or the Cl^--sufficient controls, could be kept without significant changes of properties for repeated withdrawals of aliquots as needed during the course of a set of thermoluminescence measurements. The approximate duration of an experimental run, in which 1–6 flashes were given, was 40 min.

In a normal experiment, 90 μl of the ice-cold suspension was withdrawn and applied to a 2 cm × 2 cm filter paper mounted on a precooled, heatable metal stage. After having been covered with a transparent plastic plate, the sample was usually cooled to 3–5°C, illuminated with one or more flashes spaced 0.7 s apart, and then quickly (within approx. 2 s) immersed in liquid N_2.

Three protocols were developed for addition of

Cl^- after flashing. In one, the membrane suspension was illuminated while still in the clear plastic tip of the 'Pipet Man' (model P-200) pipet. It was then applied to a filter paper that had been soaked in NaCl solution and dried to provide, when completely redissolved, sufficient NaCl to the sample for a final concentration of 70 mM. Alternately, the flashed sample in the pipet tip was quickly injected into an ice-cooled test-tube containing 3 μl 2 M NaCl and then withdrawn either for immediate application to the filter paper, or for further flash exposure and subsequent application. Controls were handled identically, but the tubes contained 3 μl deionized water. The maximal time that was permitted to elapse between the initial flashing and immersion into liquid N_2 was about 30 s. Considering the initial temperature of the sample being 0°C, and its recooling during mixing with NaCl, its temperature should have remained below 10°C throughout the handling. In a third approach (Fig. 5), all treatments were performed in a cold room (5°C), and the N_2-immersed sample was then carried for assay to the thermoluminescence detection system. The detection system has been described elsewhere [12]. The average heating rate was about 1°C/s in all experiments, except the ones in Fig. 5 when the rate was 0.75°C/s.

Results

Principles of thermoluminescence measurements

Thermoluminescence emission from flash-illuminated photosynthetic material can, in principle, provide two types of information about PS II [12–15]. First, its intensity depends, among other things, on the number of thermally induced charge recombination events which, in turn, is determined by the nature and abundance of participating electron acceptors and donors. The sources of electrons in the thermoluminescence-generating recombination reaction are Q_B^- in the absence, and Q_A^- in the presence of DCMU. The abundance of the former varies with a periodicity of 2 upon illumination with 1, 2, 3, 4, etc., flashes. Superimposed on such periodic variations is the periodicity of the oxidant storage for water oxidation which involves the stable S_0 and S_1 states, and the unstable, i.e., easily rereduced, states S_2 and S_3. The overall consequence is an oscillation of the thermoluminescence with usually a minimum after $n \times 4$ flashes [12] (see also Figs. 1, 4 and 6).

Another important parameter is the temperature at which the maximal luminescence occurs. It is an approximate measure of the free energy of activation that has to be supplied to allow a recombination of stored charges to re-excite the reaction center pigment and, thence, the antenna chlorophylls [13–15]. This free energy of activation can be related directly to the difference between the energy of the excited reaction center chlorophyll, and the redox potential gap separating the stored oxidant and reductant.

The measured values of both, luminescence intensity and peak temperature, depend on the experimental conditions, among them the rate of heating. The latter parameter has been taken into account in published theoretical analyses [14,15]. However, in order to permit easy comparative evaluations, one usually keeps the experimental conditions constant within a set of measurements.

Effect of Cl^- depletion on the thermoluminescence emission

Since PS II particles are optimally stable under slightly acid conditions, initial experiments were performed with suspension of pH 6. Fig. 1 depicts, among others, recordings of the thermoluminescence pattern of normal, Cl^--sufficient particles at that pH. One notes the typical oscillation of the luminescence intensity with the number of preilluminating flashes as described above. A variation of the emission temperature is also evident, with the highest emission intensity and temperature coinciding after one or five flashes. As Fig. 1 shows, quite similar properties of the thermoluminescence were seen with Cl^--depleted preparations after readdition of the anion. Hence, in many subsequent experiments the effect of Cl^- was assessed on the basis of comparisons with the thermoluminescence of Cl^--depleted preparations after readdition of the anion.

The third series of traces in Fig. 1 represents measurements made with the Cl^--depleted PS II particles. The occurrence of a thermoluminescence emission confirmed the earlier findings [8–11] which had suggested that oxidant storage at the

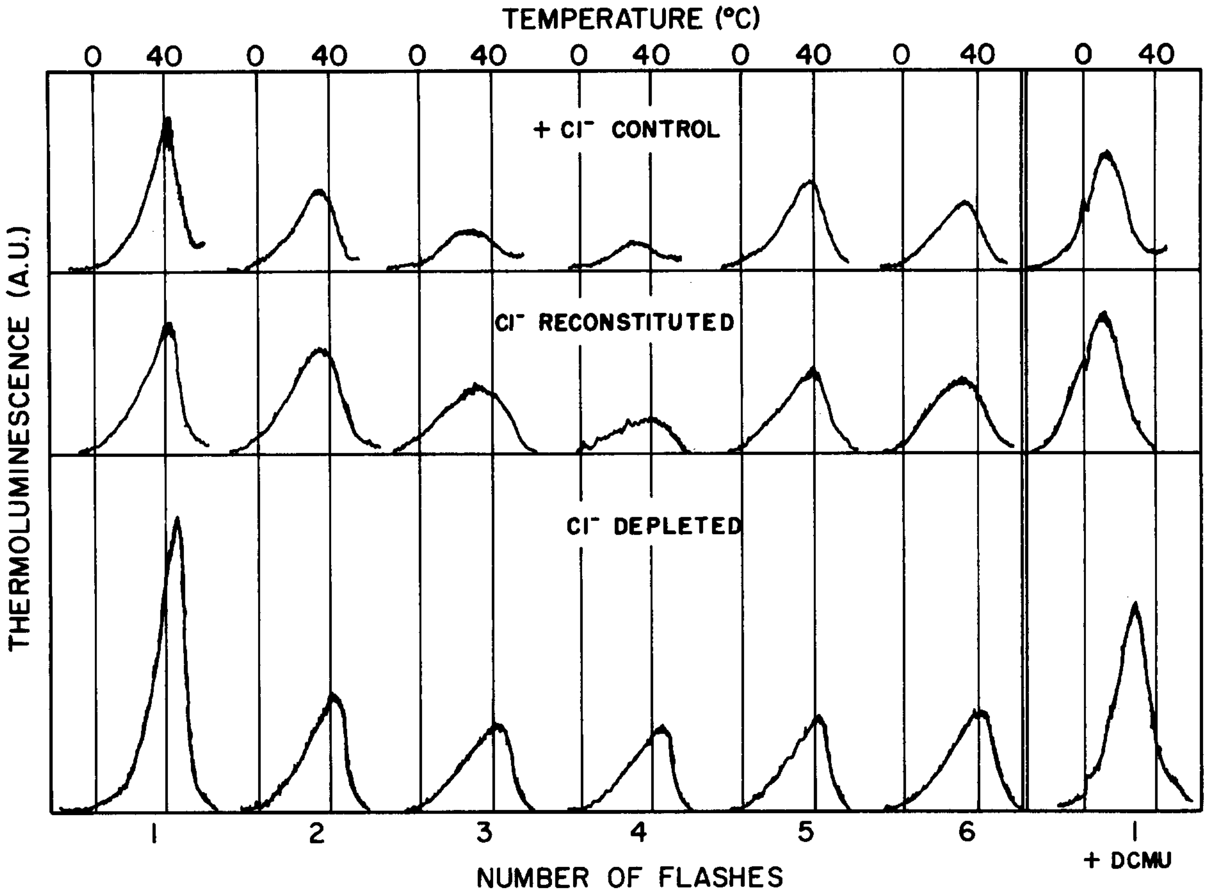

Fig. 1. Effect of Cl^- on the thermoluminescence emission at pH 6.0 elicited by illuminations with one to six flashes. Flash illumination at approx. 2°C; media contained 10 mM Na_2SO_4 and 400 mM sucrose. Cl^- depletion was by 8 s exposure to 10 mM Na-CAPS (pH 9.7) after which 65 mM Na-Mes was injected. For reconstitution 30 mM NaCl were added after depletion. Control samples were not pH shocked and supplemented with 30 mM NaCl in an Na-Mes buffered medium. 15 μM DCMU were added before the flash illumination when indicated to be present. A.U., arbitrary units.

water-splitting site is possible after removal of Cl^-. The change of the luminescence intensity of the sample, when illuminated with two flashes rather than one, indicated that a stable oxidant was generated by both flashes. Additional flashes, however, elicited luminescence bands that were almost identical to the one seen after two flashes. This strongly suggested that the charge-accumulating process was arrested after the action of the second flash, i.e., after generation of the S_3 state.

Other effects of Cl^- deficiency on the thermoluminescence were an unusually high emission intensity of samples preilluminated by one flash, and the elevated emission temperatures. The latter response was particularly evident for measurements made after two or more flashes. This indicated a higher than normal free energy of activation for especially the $S_3Q_B^-$ recombination reaction.

Fig. 1 also displays recordings made with preparations poisoned with DCMU. These measurements were designed to tell us whether the altered properties of the thermoluminescence in the absence of Cl^- could be attributed to the oxidizing side of PS II. The presence of DCMU would not be expected to affect the oxidant, but replace Q_B^- with Q_A^- as the partner in the recombination reaction. It can be seen, that an upward shift of the emission temperature occurred also under such conditions. This result placed most, if not all, actions of Cl^- on the oxidant side of PS II, in agreement with earlier studies on the Cl^- action [2]. The observed effect of Cl^- deficiency on the thermoluminescence can be contrasted with the effect of bicarbonate depletion which largely influenced the reducing side of PS II [20]. In this case the emission temperature was also higher but only in the absence of DCMU.

The rather high emission temperature measured with control preparations illuminated with one flash at pH 6 made it difficult to assess the effect of Cl^- deficiency on the $S_2Q_B^-$ recombination. From experiments with intact thylakoids it is known that this elevated emission temperature is restricted to slightly acidic media [21]. Therefore, we recorded the thermoluminescence pattern of PS II particles over a wide pH range with or without preceding removal of Cl^-. Fig. 2 summarizes the results of measurements made after preillumination with one or two light flashes in the presence and absence of DCMU. It can be seen that in the absence of the inhibitor, and after one flash, an increase of the pH above 6 did indeed move the emission temperature for Cl^- sufficient samples towards that recorded after exposure to two flashes. In fact, the emission temperature at pH > 6.5 was essentially independent of the number of flashes given. For reasons not quite clear at this time, the same became true at pH ⩽ 5.5 when the emission after two flashes peaked at a temperature as high as that seen for the emission after one flash at pH ⩽ 6.

In the absence of Cl^-, the emission temperature remained high, regardless of the pH and the preillumination. A corresponding upward shift of the emission temperature throughout the tested pH range was also evident for Cl^--deficient membrane particles poisoned with DCMU. The smaller magnitude of this shift at low pH can perhaps be attributed to a less efficient creation of a Cl^--depleted state, and at pH 8 to the known instability of Cl^--depleted particles at such elevated pH.

At neutral or slightly alkaline pH where for unpoisoned preparations the differences between the emission peak temperatures were maximal regardless of the flash number, a clear separation was often evident of two emission peaks. One was elicited at a temperature identical, or almost identical to that of Cl^--sufficient preparations, and the other at the temperature typical for Cl^--deficient samples. In Fig. 3 a series of recordings is shown which documents how on the single emission band obtained under severe Cl^- deficiency a new one was superimposed and eventually became dominant as Cl^- was made less limiting. However, the reconstituted control obviously did not fully regain the oscillatory behavior of its thermoluminescence. This could be due to a suboptimal Cl^- concentration, or to the lengthy storage of the sample under Cl^--deficient conditions which was required for completion of this series of measurements. But it is evident that some degree of normal oscillations was reintroduced even in this case.

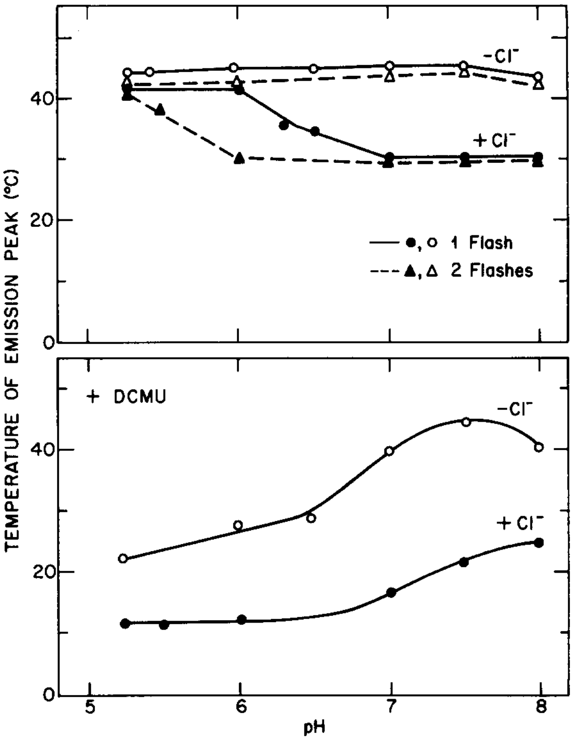

Fig. 2. pH dependence of thermoluminescence emission temperatures for Cl^--deficient and Cl^--sufficient PS II membrane particles. Cl^- depletion and other experimental conditions as for Fig. 1, except that at pH ≥ 7.5 the alkaline shock was substituted for by addition of 60 mM Na_2SO_4, and that different buffers were used. Mes for pH 5.3–6.5; Mops for pH 7, and Hepes for pH ≥ 7.5. The $+Cl^-$ samples contained 50 mM NaCl for pH < 7, and 75 mM NaCl for pH ≥ 7.5.

'Complete Cl^- deficiency' in the experiments of Fig. 3 was accomplished by adding relatively high concentrations of Na_2SO_4 [19]. Since this method

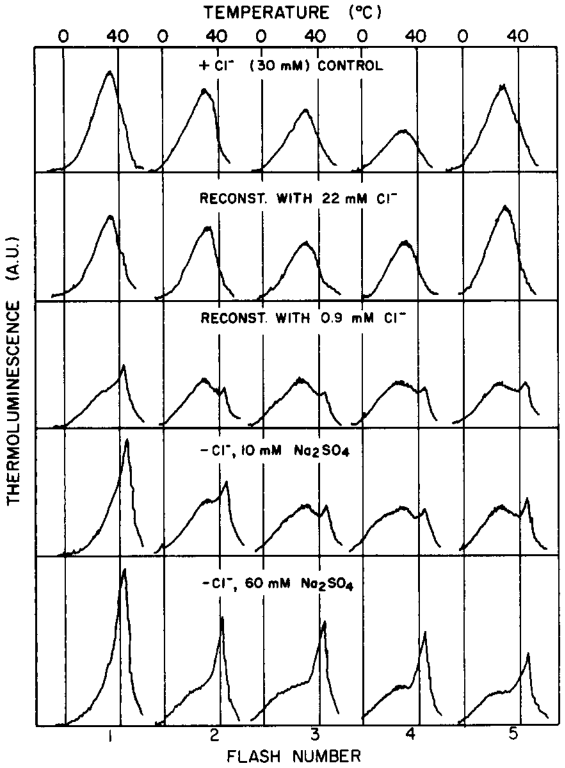

Fig. 3. Thermoluminescence at pH 7.5 after 1–6 flashes as function of Cl^- content. Experimental conditions, see legend Fig. 2, and information in the figure. A.U., arbitrary units.

of Cl^- removal was used exclusively in the tests described below, we made certain in separate experiments that the thermoluminescence pattern measured by us at pH 7.5 in the presence of Na_2SO_4 was qualitatively identical to that obtained when Cl^- removal had been accomplished by an alkaline shock in complete absence of SO_4^{2-}. The traces of the relevant experiment are not shown, and neither is a test revealing that Cl^--free conditions at pH 5.5 resembling those seen after alkaline shock could be obtained by preincubation of the sample with 70 mM Na_2SO_4 at pH 7.5, and a subsequent pH change.

Reversibility of the property changes of the oxidants stored in a Cl^--depleted PS II

The abnormal thermoluminescence emission from Cl^--depleted PS II particles raised the question whether Cl^- deficiency caused an abnormal set of reactions, or whether it merely changed the properties of S_2 and S_3. In the first case, water oxidation would fail to occur because the charge accumulating sequence proceeded in an abortive direction. In the second scenario, the property change may be readily reversible when Cl^- is made available.

To test the latter possibility, several approaches were taken. In one, a Cl^--free suspension of PS II particles was treated with 70 mM Na_2SO_4 at pH 7.5 to displace all Cl^- from the water oxidase. Thermoluminescence measurements were made on individual samples after illumination with various flash regimes, and Cl^- was either withheld throughout the procedure or added immediately after the last flash exposure. In another experimental run, Cl^- was supplied directly to the sus-

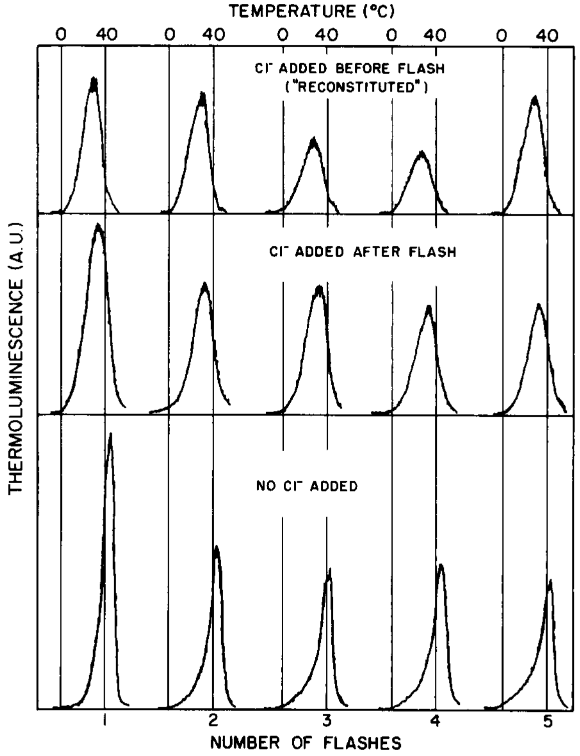

Fig. 4. Reconstitution of Cl^--depleted PS II membrane particles by addition of Cl^- after flash illumination, or before. Depletion procedure by addition of 60 mM Na_2SO_4 to a Cl^--free suspension medium containing 400 mM sucrose and being buffered with 30 mM Na-Hepes at pH 7.5. Post-flash reconstitution was accomplished by applying the sample to NaCl carrying filter paper (see Materials and Methods). Pre-flash reconstitution was done by adding to the stock-suspension 50 mM NaCl. A.U., arbitrary units.

pension, and the thermoluminescence of such 'reconstituted' preparation was recorded. The results are shown in Fig. 4. It can be seen, that addition of Cl^- after the flash(es) resulted in thermoluminescence bands which peaked at a temperature that was almost as low as that of the emission bands of the reconstituted preparation. This suggested a reversal of the S-state properties of the Cl^--depleted samples to those of normal ones. As was expected, the oscillation of the thermoluminescence intensity following the post-flash reconstitution remained arrested after the second flash.

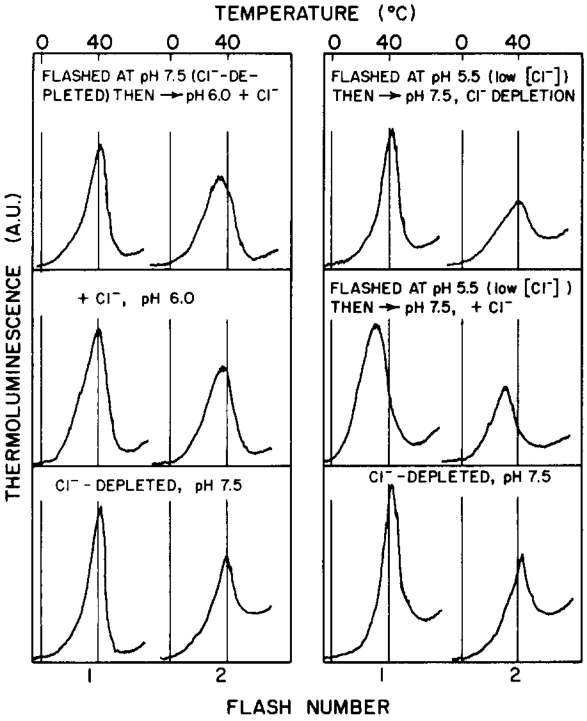

Fig. 5. Reconstitution with Cl^-, or removal of Cl^-, after flash illumination of PS II membrane particles, with simultaneous change of pH. Flash illumination at 5°C ambient temperature as described in Materials and Methods. Left panel: depletion with 50 mM Na_2SO_4 in Na-Hepes buffered medium at pH 7.5 containing 400 mM sucrose and 0.3 mM NaCl. Reconstitution accomplished by adding 50 mM NaCl plus sufficient Mes to attain pH 6.0. Right panel: samples in Na-Mes-buffered medium at pH 5.5 containing 400 mM sucrose and 0.4 mM NaCl; pH change to pH 7.5 accomplished by addition of 80 mM Na-Hepes containing either 100 mM Na_2SO_4 for depletion or 50 mM NaCl for reconstitution. Recordings shown in lowest section were flash illuminated at pH 7.5. Other information given in the figure. A.U., arbitrary units.

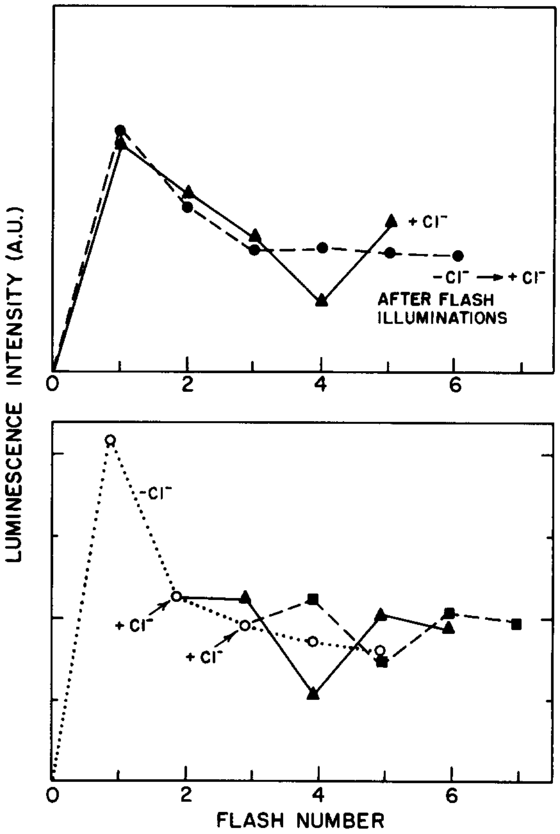

Fig. 6. Response of the patterns of thermoluminescence band heights to reconstitutions of Cl^--depleted samples at different times during the flash sequence. Medium containing 400 mM sucrose, 60 mM Na_2SO_4, 40 mM Na-Hepes (pH 7.5). Samples flashed in pipet and reconstituted with NaCl to give 65 mM as described in Materials and Methods. The $+Cl^-$ control of the upper panel had received 65 mM NaCl by addition to the stock suspension. A.U., arbitrary units.

In order to prove unambiguously the regeneration of a normal S_2 state by Cl^- addition, advantage was taken of the fact that, as discussed earlier, at pH 6.0 the thermoluminescence of Cl^--sufficient samples peaked at different temperatures when the preillumination was with one or two flashes. Since, on the other hand, Cl^- depletion and repletion was more readily accomplished at pH 7.5, we exposed samples after incubation with Na_2SO_4 at pH 7.5 to one or two flashes, and subsequently added NaCl while simultaneously changing the pH to 6.0. The thus reconstituted preparations, now at a lower pH, were then frozen and their thermoluminescence measured. The observed low emission temperature after two flashes, in contrast to the higher one after one flash, as documented in Fig. 5, clearly supported the interpretation of the other experiments that a state equivalent to the normal S_3 could be formed in Cl^--depleted PS II preparations.

The restoration of normal luminescence emissions simply by Cl^- addition suggested spontaneously reversible changes in Cl^--deficient units. To test whether these changes could be induced also in the other direction, namely on an S_2 state formed under Cl^- sufficient conditions, we added 50 mM Na_2SO_4 to a once flashed Cl^--sufficient control sample. As can be seen from the temperature shift of the thermoluminescence recorded in Fig. 5, Cl^- deprivation after flashing did indeed induce thermoluminescence properties typical of Cl^- deficient PS II units.

From the view that had emerged about the role of Cl^- in PS II, the prediction could be made that readdition of Cl^- to a twice-flashed Cl^--depleted sample would return it to conditions which should allow a normal resumption of charge accumulation and water splitting. Using the indirect approach of thermoluminescence measurements, we were able to support this prediction fully. As can be seen from the data shown in Fig. 6, a normal oscillatory pattern could be elicited following Cl^- addition to Cl^--depleted preparation after illumination with two flashes. However, when Cl^- addition was preceded by three flashes, the subsequent oscillation of the thermoluminescence was delayed by one flash. This result can only be interpreted by a block in the $S_3 \rightarrow S_4$ transition, and rules out the formation of an abnormal S_4 state that, in the absence of Cl^- fails to regenerate S_0 because it is unable to oxidize water.

Discussion

When interpreting our thermoluminescence measurements in the context of what is already known about the action of Cl^-, it is important to recognize that, during warming of our flash-illuminated samples, the time they spent at temperatures conducive to biochemical activity usually guaranteed attainment of equilibria. Hence, the recombinations responsible for the thermoluminescence emission must be attributed to charged intermediates of considerable stability. With regard to the thermoluminescence bands analyzed in this study, these intermediates presumably are the oxidizing S_2 or S_3 states of the water oxidase and the reduced acceptors Q_B^- or, in DCMU-inhibited preparations, Q_A^-.

On this background we conclude that Cl^- removal did not prevent the light-dependent advances of the S_1 state of dark adapted PS II units to the S_3 state. However, the S_2 and S_3 states formed in the absence or presence of Cl^- have different properties which are reversibly interconvertible. Since Cl^- deprivation prevents oxidant accumulation beyond the S_3 state, and thus blocks the water-oxidizing step, we may assume that the altered nature of the S_3 state precluded acquisition of an additional oxidizing equivalent. Such specific sensitivity of the $S_3 \rightarrow S_4$ transitions is known also from other treatments, e.g., an exposure to alkaline conditions [22], as well as a removal of Ca^{2+} [23] or of the 33 kDa extrinsic polypeptide [17].

The picture that emerges from our measurements agrees with Sinclair's [11] interpretation of his O_2 measurements in which he postulated that Cl^- is necessary for the $S_3 \rightarrow S_4$ transitions. However, it seems to disagree with the fluorescence measurements of Itoh et al. [10] and Theg et al. [9] which suggested a block of the $S_2 \rightarrow S_3$ advance when Cl^- had been removed. Central to the interpretation of the fluorescence data was the assumption that of the two electrons which were successively available for P^+-680 reduction in Cl^--deficient PS II units, only one was abstracted from the S complex, thus allowing a transition $S_1 \rightarrow S_2$. The positive charge left by the other electron on the primary donor Z was not neutralized by an electron within the 60 ms separating the flashes. This suggested an inability of the S complex of Cl^- deficient water oxidases to furnish an additional reducing equivalent which usually is accomplished in less than 1 ms. Yet, most of the Z^+ eventually became reduced in a biphasic manner with halftimes of 100 ms and several seconds for the two observed phases [10]. In our experiments, the flashes were spaced almost 1 s apart, and many seconds at $T > 0°C$ were available between the light exposure and the temperature-induced recombination reaction. Hence, the charge distribution in our samples must have been quite different from that encountered in the fluorescence studies. It is conceivable that the faster of the two phases of Z^+ re-reduction after the second flash did in fact involve a transfer of an electron from the S complex. The evidence for a S_3 state in our thermoluminescence measurements, in contrast to the results of the fluorescence experiments, can therefore be attributed to a slowed rate of electron flow between the Cl^--deficient water oxidase in its modified S_2 state, and Z^+. A photooxidation of the water oxidase beyond the modified S_3, however, apparently did not occur. Hence, after multiple flashes, Z^+ should be quite stable as can indeed be inferred from the indirect measurements of Itoh et al. [10].

The relatively high temperature needed to elicit a thermoluminescence generating recombination reaction between the reductants Q_B^- or Q_A^-, and the S_2 and S_3 states of Cl^--deficient PS II units, suggested that the stability of the oxidants was greater than in normal, Cl^--sufficient preparations. Preliminary measurements of the lifetimes of the oxidants support this notion. However, the determined half lives of 150–250 s at 25°C are not quite long enough to explain a preponderance of the S_2 state in 6 min dark-adapted, Cl^--deficient thylakoids which had been deduced by Muallem et al. [7] from O_2 evolution patterns after readdition of Cl^-. It is questionable that this discrepancy can be attributed to differences between intact thylakoids and purified PS II preparations because we found the thermoluminescence response to Cl^- deficiency to be quite similar in both types of material.

Our results do, however, predict at least qualitatively the burst of light which Muallem et al. [7] saw when they injected NaCl into preilluminated suspensions of Cl^--depleted thylakoids. This sudden enhancement of the 'afterglow' would be expected as the more stable S_2 and S_3 states of the Cl^--deficient PS II units were spontaneously converted into the more readily recombining normal states. In the work of Theg et al. [9], an intense, initially rapidly decaying luminescence was seen 50 ms after illuminations of DCMU poisoned, Cl^--depleted thylakoids, in contrast to a much more slowly decaying emission from normal preparations. This result is difficult to explain if one considers that, according to our thermoluminescence measurements, the lifetimes of the $S_2Q_A^-$ and $S_2Q_B^-$ pairs in Cl^--depleted units are longer than under Cl^--sufficient conditions, and that the kinetics of Z^+ reduction by the S-complex in its S_1 state are not much affected by Cl^- removal [9,10]. Hence, a reconciliation of the thermoluminescence and afterglow measurements must await kinetic analyses of all involved oxidation-reduction events.

Our view of the role of Cl^- in the electron transfer steps on the oxidant side of PS II, as it emerges from this study, can be described schematically as in Fig. 7. Here, the abnormal S states formed in Cl^--depleted units have been symbolized as Σ states. While at this stage the evidence supports only the formation of Σ_2 and Σ_3, we have included Σ_1 because of the following two experimental results. First, a modified multiline EPR signal has been measured with Cl^--depleted thylakoids after formation of an S_2 state at 200 K [24]. Second, in preliminary tests we were able to generate a thermoluminescence band reflecting a $\Sigma_2Q_B^-$ recombination after Cl^--deficient PS II membrane particles were illuminated at 208 K. The latter result could possibly be explained by assuming that a normal S_2 was formed at the low temperature, and an $S_2 \rightarrow \Sigma_2$ conversion occurred during warming. However, the combined evidence from both types of experiment strongly suggest that a pre-existing Σ_1 state was oxidized to Σ_2 by the illumination of the frozen samples. It must be acknowledged, however, that very recent NMR studies [25] revealed high-affinity Cl^- binding in PS II particles only after one or two flashes, i.e., in the S_2 and S_3 states.

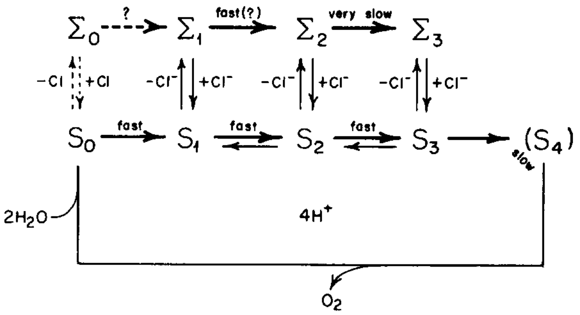

Fig. 7. Schematic presentation of our view of the action of Cl^- on the charge-accumulating sequence at the water oxidase (see text).

The upward shift of the emission temperature for Cl^--depleted preparations in both, the absence and presence of DCMU, presumably can be attributed to somewhat lower than normal oxidation potentials of the stored oxidants Σ_2 and Σ_3. These might reflect relatively subtle modifications of the redox properties of the active center of the water oxidase which is the putative site of a cluster of 2×2 Mn ions in elevated oxidation states. According to one hypothesis [1,4,26], Cl^- may in fact serve as a ligand in that cluster. However, the striking correlation of the relative effectiveness of different anions with their chaotropic character [16,29,30], may suggest more indirect actions on the protein through effects on the organization of the water molecules at the water oxidase.

It has been proposed by Homann et al. [16,28,27] and Govindjee et al. [28] that Cl^- controls the protonation states of functional groups on the water oxidase, and on adjacent polypeptides. It is noteworthy that the water oxidase does not differ from other known Cl^--requiring enzymes in that it catalyzes reactions with water as participant and responds to an addition of increasing amounts of Cl^- with a shift of its activity profile to more alkaline pH [30–32]. In other words, one effect of NaCl addition is equivalent to acidifying i.e., protonating, the domain harboring the enzyme's active site [26]. Indeed, a critical second look at the seemingly self-explanatory appearance of two thermoluminescence peaks at pH 7.5 (Fig. 3) makes questionable the interpretation that the low-temperature component is due to fully active units which had retained their Cl^-. If this were so, this component should be maximal after 1 flash, as it is in the Cl^--sufficient control, when in effect it is at its minimum, or not far from it. Interestingly, another condition under which an unexpectedly low thermoluminescence for specifically the $S_2Q_B^-$ recombination is encountered, is with Cl^- sufficient preparations at high pH [22]. What appears to be normal, undepleted units may in fact be partially depleted units which have, as a consequence, become 'alkalinized'. Thorough Cl^- removal, or its displacement by SO_4^{2-} [11], would be needed for an exclusive formation of the Σ states. This view could reconcile our measurements with the experimentally supported notion [33,34] that more than one Cl^- per water oxidase is needed for its optimal activity. It also would put into a new perspective the postulate that Cl^- anions may interact in more than one way with the protein complex that catalyzes the photosynthetic oxidation of water [4,35].

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