Biochimica et Biophysica Acta, 1100 (1992) 303–311

BBABIO 43608

Transient peroxide formation by the manganese-containing, redox-active donor side of Photosystem II upon inhibition of O_2 evolution with lauroylcholine chloride

Gennady Ananyev [a], Tom Wydrzynski [b], Gernot Renger [b], Vyacheslav Klimov [a]

[a] *Institute of Soil Science and Photosynthesis, Russian Academy of Sciences, Pushchino, Moscow Region (Russia)* and [b] *Max-Volmer Institute for Biophysical and Physical Chemistry, Technical University Berlin, Berlin (Germany)*

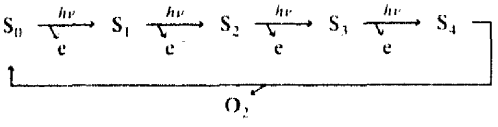
(Received 18 December 1991)

Key words: Photosynthesis; Photosystem II; Lauroylcholine chloride; Water oxidation; Peroxide formation

In an earlier study it was demonstrated that multiple electron flow through Photosystem II (PS II), including apparently the S-states, can be sustained without detectable O_2 evolution upon treatment of samples with the lipid analog compound lauroylcholine chloride (LCC) (Wydrzynski et al. (1985) Biochim. Biophys. Acta 809, 125–136). The question remained, however, as to what is the source of the electrons under this condition. In this study we extend our observations of the LCC effect and show that a peroxide-type intermediate is transiently formed on the donor side of PS II. Upon using the luminol/peroxidase method to detect peroxide, a new chemiluminescent signal (S_D) appears after the illumination of LCC-treated samples. This new signal is kinetically distinct (t_{max} = 2–3 s) from the chemiluminescent signal (S_A, $t_{max} \approx 0.2$ s) ascribable to the free H_2O_2 formed by the reduction of O_2 on the acceptor side of PS II (Ananyev and Klimov (1989) Biochemistry (USSR) 54, 1587–1597), which appears in both control and LCC-treated samples. Both S_A and S_D are sensitive to the PS II inhibitor DCMU to about the same extent. In the presence of low levels of exogenously added catalase (about 50 μg/ml), S_A can be completely eliminated but not S_D, which disappears only at higher catalase concentrations. It is suggested that the peroxide which gives rise to S_D is initially sequestered within the sample. After the complete removal of the functional manganese by a TEMED extraction procedure, S_D is lost but not S_A, with or without the addition of the artificial electron donor, hydroxylamine. In a sequence of brief (1 μs) light flashes, the progressive differences in S_D amplitudes exhibit periodic behavior, beginning on the first flash, but without a consistent pattern. As the flash sequence advances, a consumption of the peroxide appears to take place. The results are consistent with the hypothesis that in LCC-treated samples, the water-splitting catalytic complex is perturbed in such a way so that the substrate water molecules are not fully oxidized to O_2.

Introduction

The molecular mechanism by which water is oxidized to O_2 in Photosystem II (PS II) of chlorophyll *a* (Chl *a*)-containing organisms is for the most part unknown. Nevertheless, the formation of the O–O bond is likely to pass through a bound, peroxidic type of intermediate at some point in the reaction sequence [1]. In the well-established S-state scheme developed by Kok and co-workers [2,3], an O_2 molecule is released only after the co-operative interaction of four oxidizing equivalents, generated by sequential photoactions at the PS II reaction center, i.e.,



In the Kok scheme, the entrance of the two substrate water molecules and the release of the water protons remain largely under debate (for a discussion, see Refs. 4 and 5) and are omitted in the above formulation. However, with regard to the O–O bond formation, two broadly defined types of mechanism may be considered: (1) concerted-oxidation reactions, in which the two substrate water molecules are oxidized simultaneously during the final $S_3 \rightarrow S_4 \rightarrow S_0 + O_2$ transition; and (2) step-oxidation processes, in which bound water

Abbreviations: Chl *a*, chlorophyll *a*; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethyl urea; LCC, lauroylcholine chloride; Mes, 2-(*N*-morpholino)ethanesulfonic acid; P680, primary electron donor in PS II; S_A and S_D, chemiluminescent signals; TEMED, *N,N,N',N'*-tetramethylethylenediamine.

Correspondence to (present address): T. Wydrzinski, Research School of Biological Sciences, The Australian National University, GPO Box 475, Canberra, ACT 2601 Australia.

becomes partially oxidized at an S-state prior to the final transition. A distinguishing feature between these two types of mechanism would be the lifetime of the expected peroxidic intermediate. In the former case, the peroxidic intermediate would exist only during the O_2 release time of the final transition, now generally agreed to be within a few milliseconds [6–12], while in the latter case, the peroxidic intermediate would exist during one of the earlier S-states, where the half-life times for S_2 and S_3 states can vary from several seconds to tens of minutes depending upon the temperature and pH [3,6,13–16] and the S_1 state is dark stable [13,17,18]. In all mechanisms, at ambient temperatures the proposed peroxidic intermediate would most likely exist within a dynamic equilibrium between bound water molecules and either the oxidized catalytic metal center [19] or another redox-active ligand within the protein matrix [20,21].

Although the various S-states can apparently be forced to interact with added H_2O_2 [22–28], under normal conditions H_2O_2 by itself does not appear to compete efficiently with water as an electron source [29]. Likewise, there is as yet no direct evidence to indicate that a bound or free peroxidic intermediate actually forms during the normal S-state cycling of intact, highly O_2-evolving samples. On the other hand, there is accumulating evidence to suggest that H_2O_2 can be produced when the PS II complex is perturbed in some way, for example, by adding an ADRY reagent [30], upon removal of the so-called extrinsic proteins [23,24], under osmotic and/or chloride stress [31,32] or after acid denaturation [33,34]. The site at which H_2O_2 is formed in PS II under these various conditions is at present controversial, whether it is on the acceptor side [35] or the donor side [24,31,32,34] or whether the H_2O_2 arises from reactions at the catalytic site itself [31,32,34] or at a functionally displaced manganese center [24]. In view of the complex nature of PS II, it would not be surprising to find that all of these possibilities may be involved, depending upon how the PS II complex is perturbed and the type of assay system that is employed.

Regardless of the conditions used, when H_2O_2 production is observed, it is usually found superimposed over a residual (and presumably a normally produced) O_2 evolution background. Since PS II samples possess to various extents an inherent catalase activity, the separation of the H_2O_2 production from the residual O_2 evolution often becomes experimentally difficult. Likewise, for thermodynamic and/or kinetic reasons, not all peroxidic intermediates formed in PS II may be released into the external aqueous phase [36], which would be required for many peroxide detection assays. Consequently, less effort has been made on investigations of this aspect of PS II than would otherwise normally be attempted.

Within the past few years, an unusual inhibitory condition of PS II has been reported by Wydrzynski and co-workers using the lipid analog compound, lauroylcholine chloride (LCC). It was found that, upon treating PS II samples with limited amounts of LCC under certain experimental conditions, all detectable O_2 evolution activity can be completely suppressed, even though the measured electron flow through 30% to 50% of the PS II centers remains normal, under both continuous and multiple flash illumination and without the addition of artificial electron donors [37,38]. The important question in these observations has been, of course, what is the source of the electrons (and its oxidation product(s)) in those PS II centers which continue to turnover. In this communication, we now provide evidence to show that after LCC treatment the donor side of PS II is capable of transiently producing what appears to be a sequestered form of peroxide. The results are consistent with the idea that water is still likely to be the electron source under this condition and sustains the multiple electron flow through PS II without O_2 evolution. The implications of these findings in terms of the native water-splitting mechanism are discussed briefly in this communication and will be expanded further in a future publication.

Experimental

Photosystem-II-enriched membrane fragments were prepared from hydroponically grown spinach according to a detergent solubilization procedure similar to one described earlier [39] and using a Triton X-100 to chlorophyll ratio of 20:1. After the detergent treatment, the samples were washed four times in a medium consisting of 20 mM Mes/NaOH (pH 6.5), 35 mM NaCl and 300 mM sucrose. The samples were then stored at 1 mg Chl/ml in liquid nitrogen, in which case the final suspension medium contained 10% glycerol as a cryoprotectant.

After storage, the frozen PS II samples were thawed and washed once in a medium containing 10 mM Mes/NaOH (pH 6.5) and 10 mM NaCl. For treatment with lauroylcholine chloride (LCC), the samples were suspended to 200 μg Chl/ml in the same medium to which LCC was added as a small aliquot in a ratio to the chlorophyll of 3:1 (w/w). The samples were allowed to incubate in the dark at room temperature (about 20°C) for 5 min with gentle shaking before being centrifuged. The pelleted sample was then resuspended in the same buffer medium to the appropriate chlorophyll concentration. Stock LCC solutions were made in pure ethanol. The final ethanol concentration in the sample did not exceed 2%. All controls went through the same procedure, but without LCC.

To extract the manganese from PS II, samples at 0.25 mg Chl/ml were incubated in a medium consist-

ing of 20 mM Mes/NaOH (pH 6.5), 0.5 M $MgCl_2$ and 20 mM *N,N,N',N'*-tetramethylethylenediamine for 5 min on ice in the dark and centrifuged. The samples were then washed three times in 20 mM Mes/NaOH (pH 6.5) plus 10 mM NaCl. After this treatment, more than 97% of the manganese was removed from the samples as determined by atomic absorption spectroscopy. The extracted samples showed neither O_2 evolution activity nor the thermoluminescence band arising from $S_2Q_B^-$ charge recombination (data not shown).

O_2 flash yield measurements were made using a laboratory-built, Clark-type electrode that was equipped with a special Teflon membrane (Fum, CIS) which could be stretched to a thickness of about 1 μm. About 20 μl of a sample at 250 μg Chl/ml was layered into a chamber 0.3 mm thick over the membrane. The samples contained 0.4 mm $K_3Fe(CN)_6$ and 0.4 mM 2,5-dichloro-*p*-benzoquinone as electron acceptors. Saturating light flashes (FWHM = 1 μs) were obtained from a xenon flash lamp at a repetition rate of 0.5 Hz. The O_2 signals were amplified by a laboratory-built, low-impedance circuit and integrated over a time constant of 0.5 s.

The instrument set-up used to measure the luminescence from the luminol/peroxidase method of detection for peroxide [40] has been described earlier [34]. The assay medium consisted of 50 mM phosphate buffer (pH 8.0), 20 units/ml of horseradish peroxidase (purchased from Serva, Germany) and 20 μg/ml luminol (the luminol preparation contained an equal mixture of the two keto-isomeric forms). Stock solutions of luminol were prepared in DMSO and of horseradish peroxidase in phosphate buffer. For most measurements, 50 μl of the samples at 1 mg Chl/ml were rapidly injected into 1 ml of the assay medium 10 s after the illumination treatment. Illumination was performed in a plastic pipettor tip with either continuous white light for 10 s or a sequence of brief (1 μs), saturating light flashes (1–10 flashes, given at a 0.5 Hz repetition rate). The resulting luminescence was measured at 425 nm.

The room-temperature, manganese(II) 6-line EPR signals were measured using a Bruker ER 200 D instrument (X-band, 9.7 GHz), as described earlier [41]. The low-temperature (liquid helium), manganese multiline and $g = 4.1$ EPR signals were measured on a similar instrument, but equiped with an Aspect 2000 minicomputer and an Oxford Instruments ESR 900 helium flow cryostat, as also described earlier [42]. Samples were treated with LCC as explained above, but concentrated to 1 mg Chl/ml for the room-temperature measurements or to 10 mg Chl/ml for the low-temperature measurements. The samples were kept dark-adapted at all times, except for the low-temperature measurements where the samples were subjected to 5 min, continuous illumination at 200 K. In this case, the multiline and $g = 4.1$ signals are displayed from the light-minus-dark spectra.

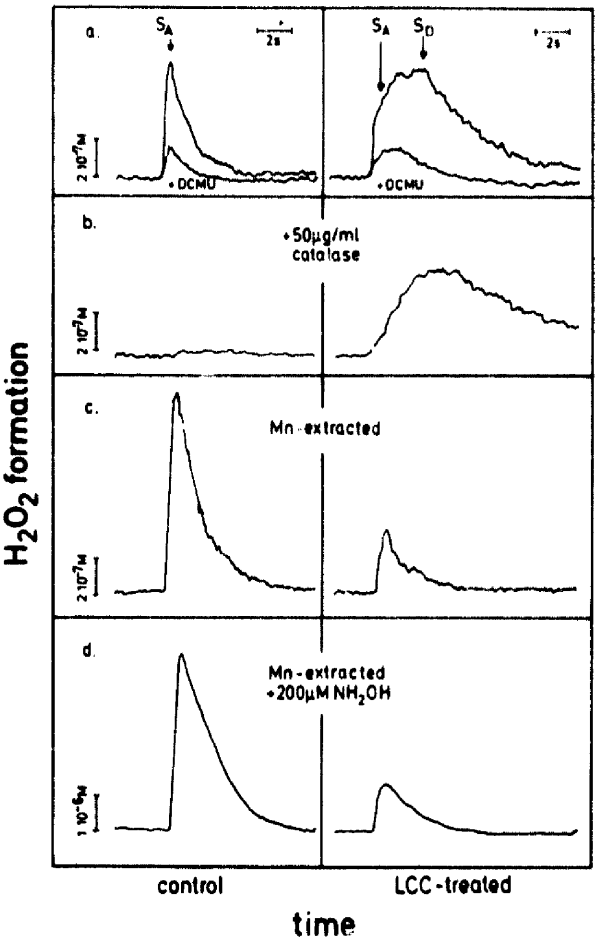


Fig. 1. Chemiluminescence measurements of peroxide formation by PS-II-enriched membrane fragments before and after treatment with lauroylcholine chloride (LCC) at LCC/Chl = 3:1 (w/w) under various experimental conditions. (a) With or without 50 μM DCMU; (b) with 50 μg/ml bovine liver catalase; (c) after removal of the functionally bound manganese by a TEMED extraction procedure (see Experimental for details); (d) samples from (c) to which 200 μM NH_2OH was added as an electron donor. All chemiluminescence measurements were determined by a luminol/peroxidase assay method as described in Experimental. The samples were illuminated in continuous white light for 10 s and then rapidly injected into the assay medium at 10 s after the end of the illumination period.

Lyophilized bovine liver catalase was purchased from Sigma, USA.

Results

After a sample of PS-II-enriched membrane fragments is illuminated with continuous light for 10 s in the absence of an added electron acceptor, a chemiluminescent signal appears upon its rapid injection into a luminol/peroxidase assay mixture. This signal, which we designated earlier as S_A [33], is shown for an untreated control sample in Fig. 1a. The signal peaks around 0.2 s after injection, although its rise kinetics

are largely distorted by the injection procedure. A standard H_2O_2 solution, when injected directly into the assay mixture, will produce an identically shaped signal, while a thoroughly dark-adapted PS II preparation which has not been preilluminated will not produce any chemiluminescence (see Refs. 33, 34).

The magnitude of S_A as shown in Fig. 1a represents about $6 \cdot 10^{-7}$ M H_2O_2. This amount would correspond to about 1 H_2O_2 molecule produced over the 10 s illumination period per two PS II reaction centers. Although this is a relatively small amount, the magnitude of S_A can vary. This will depend upon the length of the illumination time (e.g., for the PS II preparation used in Fig. 1, the S_A magnitude increases linearly with the time of illumination up to 30 s, after which the signal size becomes saturated – data not shown) and upon the extent of an inherent catalase activity that is characteristic for each sample preparation (e.g., again for the PS II preparation used in Fig. 1, after a 10 s illumination period, the S_A magnitude decreases hyperbolically with the dark time prior to injection until about 60 s, at which S_A can no longer be detected – data not shown). Nevertheless, as also indicated in Fig. 1a, S_A is most sensitive to DCMU when the inhibitor is added prior to the illumination period. This result shows that the formation of S_A is for the most part dependent upon PS II electron transport through the Q_B site. In earlier reports it was demonstrated that S_A arises from free H_2O_2 formed by the reduction of O_2 on the acceptor side of PS II in the absence of an added electron acceptor [33,34,43], as expected from thermodynamic considerations [30].

Upon treatment of PS-II-enriched membrane fragments with lauroylcholine chloride (LCC) at an amount equivalent to LCC/Chl = 3:1 (w/w) in a low salt, low osmotic medium, the chemiluminescence appears as shown for the LCC-treated sample in Fig. 1a. In this instance, there is a rapid initial rise to about 0.2 s and then a slow increase which peaks around 2 s after injection. At lower LCC concentrations the rapid initial rise may dominate the chemiluminescence while the slow increase may appear only as a descending hump in the decay of the signal. The observed chemiluminescence behavior is not a consequence of saturating the signal detection system. As shown earlier, the sensitivity of the luminol/peroxidase method is virtually linear below 1 mM H_2O_2 [40]. Also, as shown in Fig. 1a, the chemiluminescence kinetics of LCC- treated samples are essentially the same in the presence of DCMU, where the signal magnitude has been decreased by more than two-thirds. Thus, all results suggest that, in addition to S_A, a new, kinetically slower signal is created after LCC treatment. We designate this new signal as S_D.

That the observed chemiluminescence arising after LCC treatment represents two distinct signals is further indicated by the data shown in Fig. 1b. In these measurements a small amount of catalase (50 μg/ml) was added to the samples prior to the illumination period. As is clearly evident, S_A is completely suppressed when this amount of catalase is present (all other assay conditions were kept constant), whereas S_D is still observable and displays its unique slow rise kinetics.

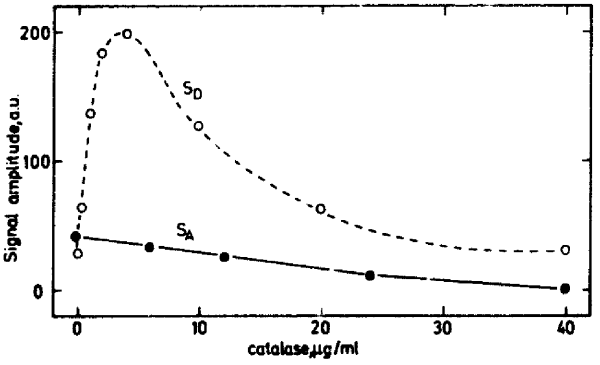


Fig. 2. The relative amplitude of the chemiluminescent signals S_A and S_D from control and LCC/Chl (3:1)-treated PS-II-enriched membrane fragments, respectively, measured as a function of the catalase concentration added prior to the illumination period. The methods of illumination and chemiluminescence detection were the same as those used for the measurements shown in Fig. 1.

The response of S_A in control samples and S_D in LCC-treated samples to a range of added catalase concentrations is shown in Fig. 2. For S_A there is a hyperbolic decrease with increasing catalase concentrations, as might be expected; but surprisingly, for S_D there is a dramatic transient increase before this signal, too, is lost at higher catalase concentrations. This unusual behavior of S_D is strictly dependent upon the enzymatic activity of the catalase. For catalase irreversibly inactivated by 1,2,4-aminotriazole and thoroughly dialyzed to remove the inhibitor, there are no changes in the S_D magnitude (data not shown). The implications of these findings will be discussed below, but the eventual loss of S_D at higher catalase concentrations indicates that it, too, probably arises from a peroxide-type intermediate.

The important question arises as to whether S_D represents peroxide formation on the acceptor side or the donor side of PS II. To determine this, the PS-II-enriched membrane fragments were first depleted of the functionally bound manganese by a TEMED extraction procedure (see Experimental). Although this procedure consistently removes more than 95% of the manganese, it is not any more damaging to PS II than are the usual NH_2OH and Tris extraction procedures, since TEMED-extracted samples can be photoactivated in O_2 evolution [44]. As shown in Fig. 1c, only S_A is observed after the TEMED treatment, regardless of

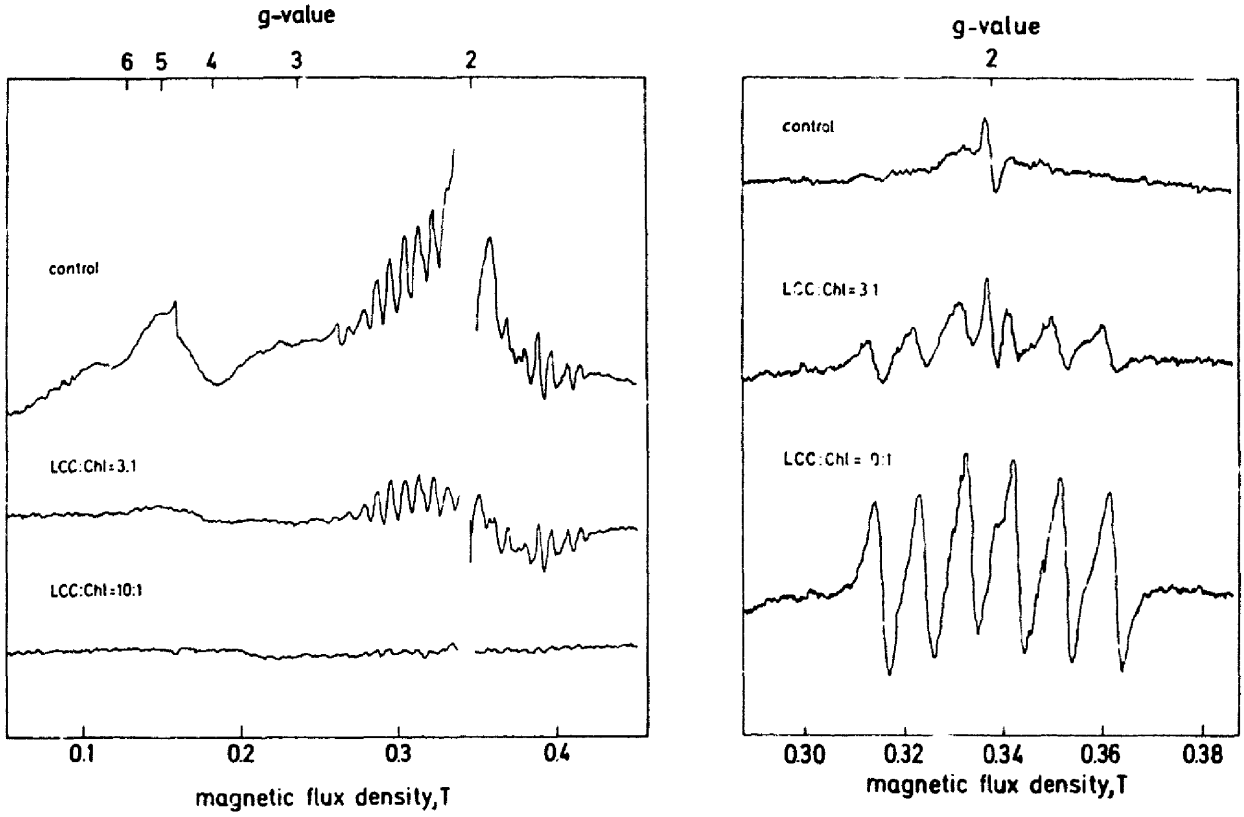


Fig. 3. Low temperature (left) and room-temperature (right) EPR measurements of the manganese in PS-II-enriched membrane fragments, before and after LCC treatment. The EPR operating conditions are referenced in Experimental and the definitions of the signals are given in the text. The amplitude of the 6-line EPR signal at room temperature at LCC/Chl = 10:1 is equivalent to that obtained after heat-inactivation of the control.

whether or not the samples are further subjected to an LCC treatment. The somewhat larger S_A magnitude of the control sample under this condition may be due to a suppression of the inherent catalase activity of PS II by the TEMED procedure or to an increase in the O_2 penetration at the acceptor side. On the other hand,

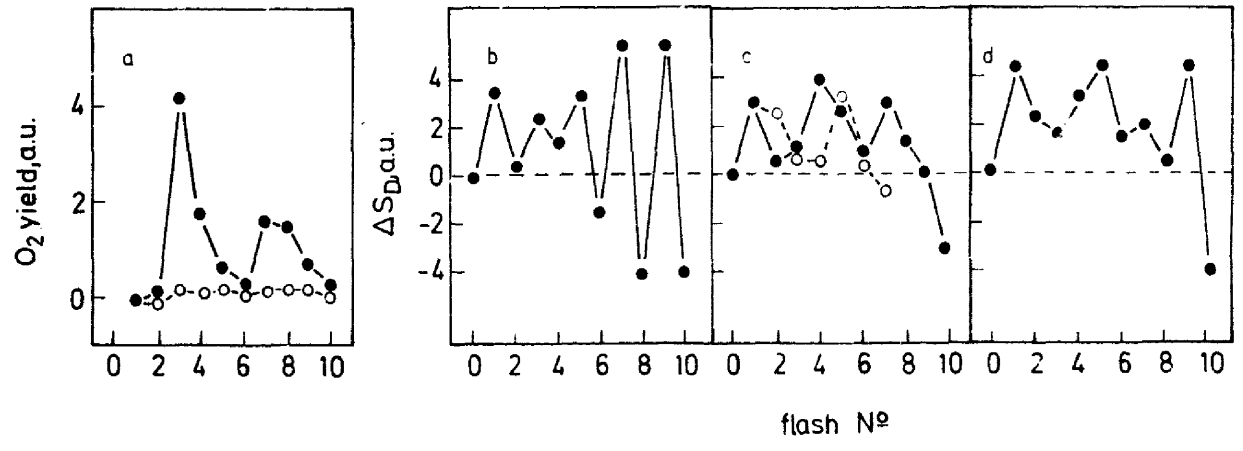


Fig. 4. The O_2 yields and the chemiluminescent signal magnitude differences (ΔS_D) measured as a function of the number of brief (1 μs), saturating light flashes of PS II-enriched membrane fragments. (a) O_2 flash yields for control (———) and LCC/Chl (3:1)-treated (------) samples. (b–d) Examples from different preparations of the ΔS_D flash yields (calculated as the difference in signal magnitude between successive measurements) after treatment with LCC/Chl = 3:1. The dashed curve (------) in (c) shows the ΔS_D flash yield pattern of the same sample after incubation on ice in the dark for 4 h. The methods of detection for the O_2 flash yields and the chemilluminescence are described in Experimental. The chemiluminescence was measured after 10 s at the end of each flash sequence for which a new aliquot of the dark-adapted sample preparation was used. The error for each data point lies within ± 10%, when measurements are repeated in rapid succession.

the significantly lower S_A magnitude of the LCC-treated sample is most likely due to the complete inactivation of part of the PS II centers by LCC (see Refs. 37 and 38). For the data shown in Fig. 1d, hydroxylamine was added to the extracted samples prior to illumination to act as an arteficial electron donor. Although the magnitude of S_A increases more than 5-fold in these measurements, S_D still does not appear after LCC treatment. Thus, it is likely that S_D requires the presence of the functionally bound manganese and does not involve the acceptor side of PS II.

The above conclusion is supported by the EPR data shown in Fig. 3. Here the room-temperature manganese 6-line signal (which indicates functionally displaced manganese ions) and the low-temperature, multiline and $g = 4.1$ signals (which indicate the functionally intact manganese ions) are shown for control and LCC-treated samples. At LCC/Chl = 3:1, where all O_2 evolution activity is suppressed, but up to 50% of the acceptor photoreduction activity remains [37,38] and S_D forms (Fig. 1), only about half of the low-temperature, multiline EPR signal is lost. The loss in the multiline signal correlates with a corresponding increase in the room-temperature, 6-line signal. Interestingly, however, more than 90% of the low-temperature $g = 4.1$ signal disappears under this LCC condition. At LCC/Chl = 10:1, where all PS II activities are inhibited [37,38], all low-temperature EPR signals are lost and the room-temperature 6-line signal maximizes, equivalent to the total, functionally displaced manganese ions as observed after heat inactivation [4i]. In a future publication we will show that S_D is completely lost under this LCC condition as well.

Since S_D seems to represent peroxide formation by a relatively intact PS II donor side in which only O_2 evolution has been inhibited, it would be important to know whether this peroxide formation exhibits a periodic behavior. To determine this, the S_D signal magnitude for samples containing a small amount of catalase (used to suppress S_A) was measured as a function of a sequence of brief (1 μs), saturating light flashes. The results are shown in Fig. 4, which presents the data as the difference in the signal magnitudes (ΔS_D) between successive flashes and also shows the O_2 flash yield measurements in presence and absence of LCC/Chl = 3:1. For untreated, control PS II membrane fragments which produce the strong periodicity of four in the O_2 flash yields, S_D does not appear. Upon complete inhibition of the O_2 flash yields by LCC:Chl = 3:1 (also measured in the presence of the catalase) (Fig. 4a), S_D does form and exhibits various flash-induced patterns as exemplified in Fig. 4b,c,d. In some cases, a strong binary periodicity can be observed (Fig. 4b). But in other measurements, apparent ternary and quaternary patterns are exhibited (Fig. 4c,d). That the periodic behavior in ΔS_D seems to be dependent upon the state of the sample at the time of measurement is indicated in Fig. 4c, where the pattern appears to become phase shifted simply by keeping the sample on ice in the dark for 4 h (dashed curve). Reproducibility of a particular data point was quite good when repeated measurements were taken in rapid succession, being well within 10%. Thus, although there does appear to be periodic behavior in the flash dependence of ΔS_D, the pattern can be highly variable. Nevertheless, in all measurements, S_D always forms on the first flash, as opposed to S_A which does not [33,34]. Additionally, as the flash sequence progresses, there are marked decreases in ΔS_D, indicating a consumption of the peroxide intermediate after certain flash-induced transitions. The significance of these observations will be discussed below.

Discussion

The unusual feature of samples treated with lauroylcholine chloride (LCC) is that even though a significant fraction (up to 50%) of the PS II centers can continue to turnover, sustaining a normal electron transport activity in the absence of added electron donors, all detectable O_2 evolution is completely suppressed. The fundamental question in this observation has been what is the source of the electrons. The compound itself, LCC, is a cationic, detergent-like molecule which is unlikely to act as an electron source and is present in too small amounts after treatment to quantitatively account for the observed multiple turnover of PS II under steady-state conditions [37]. Likewise, unpublished observations suggest that, after LCC treatment, the photooxidation of chlorophyll and carotenoid antenna pigments is not markedly enhanced. Similarly, since the electron transport kinetics of the centers which do continue to turnover without O_2 evolution appear normal [38], it is also unlikely that the protein matrix itself is being rapidly destroyed, at least during the initial part of steady-state turnover. Rather, as suggested earlier [37], it may be that water still acts as the electron source under this condition, except that it is no longer oxidized to O_2. If this is the case, then the questions arise as to what is(are) the oxidation product(s) and how has the normal water-splitting chemistry been modified such that the formation of O_2 is inhibited.

In this communication, we provide evidence to show that after LCC treatment at least a peroxide-type intermediate can be transiently produced by PS II on its donor side, when the luminol/peroxidase method of detection is employed. This peroxide formation (designated as S_D) is not only kinetically distinct from the H_2O_2 formation known to arise from the reduction of O_2 by the acceptor side of PS II in the absence of an added electron acceptor (designated as S_A, see Fig. 1a;

see also Refs. 33, 34, 43 and 45), it also exhibits a differential response to added catalase (Fig. 1b; Fig. 2). Most importantly, this peroxide formation is strictly dependent upon the presence of the PS-II-associated manganese (Fig. 1c,d; Fig. 3; see also Ref. 45). Therefore, it appears to require a relatively intact PS II donor side and could thus arise as a consequence of water oxidation.

The slower rise kinetics and differential response to added catalase of the LCC-induced peroxide formation suggest that this peroxide is not immediately released free into the surrounding aqueous medium, as is the case for the H_2O_2 produced on the acceptor side [33,34,43,45]; instead, it may be initially sequestered within the PS II complex or between the appressed regions of the aggregated material. It is known, for example, that an accessibility barrier (related to the extrinsic 33, 23 and 16 kDa proteins) does exist between added H_2O_2 and the internal redox-active components of PS II [23–28]. The failure of earlier attempts to detect externally released, free H_2O_2 in LCC-treated samples by simply adding limited amounts of catalase to the assay medium in steady-state O_2 evolution measurements [37] may be explained by such an exchange-barrier phenomenon. Alternatively, the observed peroxide formation may represent, or be mediated through, an organic free radical. But if this is the case, it is clear that the PS-II-associated manganese is involved.

The rather restricted interaction between the LCC-induced peroxide formation and added catalase as indicated above is, however, even more complex. This is indicated by the peculiar transient increase in the magnitude of S_D with increasing added catalase concentrations (Fig. 2). Although such behavior may reflect a release in the kinetic limitation by the O_2 reduction reaction on the acceptor side of PS II under continuous illumination, we note that the catalase effect is present even after a single flash. Alternatively, the transient increase may somehow be a consequence of competitive interactions for the peroxide intermediate between the sites of its formation and degradation in PS II, the added catalase and the luminol/peroxidase complex. Or it may be that the S_D signal observed in the absence or presence of catalase arise from quite different processes. The catalase effect will require further investigations.

Regardless of the complexities, the requirement of the PS-II-associated manganese in order to observe the LCC-induced peroxide formation (Figs. 1, 3) clearly indicates that the PS II donor side needs to remain relatively intact. This conclusion is supported by earlier measurements of the $P680^+$ reduction kinetics [38], where the nanosecond-range components (which reflect the coupling between the primary reactants and the water-splitting catalytic complex) were found to be present after LCC treatment in those PS II centers which continue to turnover without O_2 evolution. Likewise, as shown in this publication (Fig. 3), the hyperfine structure of the low-temperature manganese multiline EPR signal which remains after LCC treatment appears unaltered, although the $g = 4.1$ EPR signal is virtually eliminated. Thus, the water-splitting catalytic complex could be the site where the LCC-induced peroxide is formed.

According to the above suggestion, then one could a priori expect that an intermediary S-state is involved in the peroxide formation. Indeed, the formation of S_D after a single flash excitation from dark-adapted samples (Fig. 4), the presence of the normal low temperature, manganese multiline EPR signal (Fig. 3) and the occasional strong binary oscillation in the flash-induced pattern (Fig. 4b) would all be consistent with peroxide formation via the S_2 state, as has been suggested earlier [32]. But, unfortunately, the lack of consistent periodic behavior in the S_D formation (Fig. 4c,d), makes such an exact interpretation less definitive. It may be that in perturbed PS II complexes, the classically defined S-states are no longer applicable and that the observed peroxide formation arises only from an aberrant side reaction at an altered water-splitting catalytic site. This could very well explain the low quantum yield of the observed peroxide formation.

Similarly, the observed lack of a consistent oscillation pattern after flash excitation in the S_D magnitude is in apparent conflict with earlier measurements of the millisecond-range delayed light emission (DLE) in which a distinct periodicity of four was found under similar LCC inhibitory conditions [37]. The discrepancy in the two types of measurement also remains to be clarified, but an answer may lie in the origin of the different signals. For instance, it is now known that the damping parameter for the period-four oscillations in the O_2 flash yield pattern is different from the damping parameter for the DLE pattern measured for the same samples [46]. Thus, one may speculate in view of an earlier hypothesis [37] where the period-four oscillations were proposed to arise from an oxidative storage entity (which presumably would modulate the millisecond range DLE pattern) separate from a special reaction site (where presumably the O_2 or peroxide would be formed).

Another peculiar feature in the pattern of the LCC-induced peroxide formation is the large loss in the signal magnitude as the flash sequence progresses (Fig. 4). In view of the many reports in which added H_2O_2 is found to interact with the water-splitting catalytic complex (for a summary, see Ref. 47), it may not be unreasonable to expect that, due to its sequestered nature, the LCC-induced peroxide readily re-reacts with other redox active components, in either the same or neighboring centers, soon after it is formed. The

possible creation of other active oxygen species during such secondary side-reactions (e.g., superoxide anions, hydroxyl radicals or singlet oxygen) could then participate, for example, in lipid peroxidation [48] and thus be lost to detection. This could further explain why it is difficult to detect large amounts of this peroxide formation in the external aqueous medium and why there is no consistent periodic behavior. This, too, will require further investigations before more precise interpretations can be made.

In conclusion, we have shown in this paper that after treatment of PS II membrane fragments with lauroylcholine chloride, an apparently sequestered form of peroxide is transiently produced by the PS II donor side which requires the presence of the PS II associated manganese. The results support the hypothesis that water still acts as the electron source under this condition where there is no detectable O_2 evolution. The question now becomes whether this peroxide formation arises from the release of the expected peroxidic intermediate in the normal oxidation path of water to O_2, as been suggested earlier under other conditions [34] in line with a previous proposal (Ref. 36; see also Ref. 49), or whether it is actually produced by an alternative reaction path at the catalytic site, as possibly induced through an altered accessibility of the substrate water molecules. This problem will be addressed in more detail in a future publication.

Acknowledgements

We thank the Deutsche Forschungsgemeinschaft for financial support during the course of this work (Re 354/10-1), Johannes Messinger for critically reading the manuscript and S. Hohm-Veit for preparing the figures. T.W. also gratefully acknowledges travel support from the Institute of Soil Science and Photosynthesis in Pushchino and thanks Dr. Lars-Erik Andreasson, Chalmers University of Technology, Göteborg, Sweden, for his assistance in making the low-temperature EPR measurements.

References

1 Krishtalik, L.I. (1990) Bioelectrochem. Bioenerg. 23, 249–263.
2 Kok, B., Forbush, B. and McGloin, M. (1970) Photochem. Photobiol. 11, 457–475.
3 Forbush, B., Kok, B. and McGloin, M. (1971) Photochem. Photobiol. 14, 307–321.
4 Hansson, Ö. and Wydrzynski, T. (1990) Photosynth. Res. 23, 131–162.
5 Renger, G. and Wydrzynski, T. (1991) Metals Biol. 4: 73–80.
6 Joliot, P., Joliot, A., Bouges, B. and Barbieri, G. (1971) Photochem. Photobiol. 14, 287–305.
7 Bouges-Bocquet, B. (1973) Biochim. Biophys. Acta 292, 772–785.
8 Sinclair, J. and Arnason, T. (1974) Biochim. Biophys. Acta 308, 393–400.
9 Lavergne, J. (1989) Proc. Natl. Acad. Sci. USA 86, 8768–8772.
10 Jursinic, P.A. and Dennenberg, R.J. (1990) Biochim. Biophys. Acta 1020, 195–206.
11 Strzalka, K., Walszak, T., Sarna, T. and Swartz, H.M. (1990) Arch. Biochem. Biophys. 281, 312–318.
12 Mauzerall, D.C. (1990) Plant Physiol. 94, 278–283.
13 Vermaas, W.F.J., Renger, G. and Dohnt, G. (1984) Biochim. Biophys. Acta 704, 194–202.
14 Vass, I., Deák, Z. and Hideg, E. (1990) Biochim. Biophys. Acta 1017, 63–69.
15 Messinger, J. and Renger, G. (1990) in Current Research in Photosynthesis, Vol. I (Baltcheffsky, M., ed.), pp. 846–852, Kluwer, Dordrecht.
16 Messinger, J. and Renger, G. (1990) FEBS Lett. 141–146.
17 Doschek, W.W. and Kok, B. (1972) Biophys. J. 12, 832–838.
18 Velthuys, B.R. and Visser, J.W.M. (1975) FEBS Lett. 55, 109–112.
19 Renger, G. (1987) Photosynthetica 21, 203–224.
20 Matsushita, T., Spencer, L. and Sawyer, D.T. (1988) Inorg. Chem. Lett. 27, 1167–1173.
21 Renger, G. (1977) in: Topics Curr. Chem. (Boschka, F.L., ed.), Vol. 69, pp. 39–90, Springer, Berlin.
22 Velthuys, B. and Kok, B. (1978) Biochim. Biophys. Acta 502, 211–221.
23 Schröder, W.P. and Åkerlund, H.-E. (1986) Biochim. Biophys. Acta 848, 359–362.
24 Berg, S.P. and Seibert, M. (1987) Photosynth. Res. 13, 3–17.
25 Mano, J., Takahashi, M. and Asada, K. (1987) Biochemistry 26, 2495–2501.
26 Frasch, W.D. and Mei, R. (1987) Biochemistry 26, 7321–7325.
27 Frasch, W.D., Mei, R. and Sanders, M.A. (1988) Biochemistry 27, 3715–3719.
28 Sandusky, P.O. and Yocum, C.F. (1988) Biochim. Biophys. Acta 936, 149–156.
29 Velthuys, B.R. (1983) in The Oxygen Evolving System of Photosynthesis (Inoue, Y., et al., eds.), pp. 83–90, Academic Press Japan, Tokyo.
30 Sayre, R.T. and Homann, P.H. (1979) Arch. Biochem. Biophys. 196, 525–533.
31 Wydrzynski, T., Ångström, J. and Vänngård, T. (1989) Biochim. Biophys. Acta 973, 23–28.
32 Fine, P.L. and Frasch, W. (1990) in Current Research in Photosynthesis (Baltscheffsky, M., ed.), Vol. I, pp. 905–908, Kluwer, Dordrecht.
33 Ananyev, G.M. and Klimov, V.V. (1988) Dokl. Akad. Nauk, SSSR 298, 1007–1011.
34 Ananyev, G.M. and Klimov, V.V. (1989) Biochemistry (USSR) 54, 1587–1597.
35 Schröder, W.P. and Åkerlund, H.-E. (1990) in Current Research in Photosynthesis (Baltscheffsky, M., ed.), Kluwer, Dordrecht, pp. 901–904.
36 Renger, G. (1978) in Photosynthetic Water Oxidation (Metzner, H., ed.), pp. 229–248, Academic Press, London.
37 Wydrzynski, T., Huggins, B.J. and Jursinic, P.A. (1985) Biochim. Biophys. Acta 809, 125–136.
38 Eckert, H.-J., Wydrzynski, T. and Renger, G. (1988) Biochim. Biophys. Acta 932, 240–249.
39 Berthold, D.A., Babcock, G.T. and Yocum, C.F. (1981) FEBS Lett. 134, 231–234.
40 Corimer, M.J. and Prichard, P.M. (1968) J. Biol. Chem. 243, 4706–4714.
41 Wydrzynski, T., Baumgart, F., MacMillan, F. and Renger, G. (1990) Photosynth. Res. 25, 59–72.
42 Hansson, Ö., Andreasson, L.-E. and Vängard, T. (1986) FEBS Lett. 195, 151–159.
43 Shuvalov, V.A. and Krasnovsky, A.A. (1975) Biochemistry (USSR) 40, 358–367.
44 Ananyev, G.M., Shafiev, M.A., Isaenko, T.W. and Klimov, V.V. (1988) Biofizika 33, 265–269.

45 Ananyev, G.M. and Klimov, V.V. (1989) Biochemistry (USSR) 54: 1386–1393.
46 Delrieu, M.-J. and Rosengard, F. (1988) Biochim. Biophys. Acta 936, 39–46.
47 Schröder, W.P. (1989) PhD Thesis, University of Lund, Lund, Sweden.
48 Takahama, U. and Nishimura, M. (1975) Plant Cell Physiol. 16, 737–748.
49 Messinger, J., Wacker, U. and Renger, G. (1991) Biochemistry 30, 7852–7862.