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Uncoupling of detectable O_2 evolution from the apparent S-state transitions in Photosystem II by lauroylcholine chloride: possible implications in the photosynthetic water-splitting mechanism

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Recently we have introduced the use of choline/fatty acid derived compounds, in particular lauroylcholine chloride (LCC), to probe selectively Photosystem II (PS II) structure and function (Wydrzynski, T. and Huggins, B.J. (1983) in The Oxygen-Evolving System of Photosynthesis (Inoue, Y., Crofts, A.R., Govindjee, Murata, N., Renger, G. and Satoh, K., eds.), pp. 265–272, Academic Press Tokyo, Japan). In this paper we report an unusual condition in thylakoid membrane samples at relatively low amounts of LCC in which detectable O_2 evolution cannot be measured, yet electron flow through PS II is near normal without added electron donors. LCC does not appear to interfere with the O_2 yield measurements directly nor act as an electron donor itself after the Tris block. Under this condition, steady state and flash O_2 yield measurements show no O_2 release or uptake, while steady-state ferricyanide photoreduction and the variable component of the chlorophyll *a* fluorescence transient remains at more than 50% of the control. The photoreduction of the primary quinone acceptor, Q_A, measured by microsecond range chlorophyll *a* fluorescence continues for a minimum of 200 single turnover excitation light flashes. Most importantly, the yield of the 35 μs component of the chlorophyll *a* delayed fluorescence remains at approx. 65% of the control and oscillates with a normal period four over two cycles, indicating the normal cycling of the S-state transitions in PS II. Thus, it appears that PS II can operate normally without detectable O_2 evolution. The question remains as to whether water is still being photooxidized under this condition without the release of the dioxygen product, or whether there is another source of electrons. The results are interpreted in terms of the possible existence of an additional water binding component (termed 'H') in PS II and a concerted oxidation reaction mechanism for photosynthetic water splitting.

Abbreviations: Chl, chlorophyll; DCIP, 2,6-dichlorophenolindophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; F_{max}, maximum fluorescence level; F_0, instantaneous fluorescence level; ΔF, variable fluorescence ($F_{max} - F_0$); H, theoretical water-binding component; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; I_{50}, 50% inhibition point with respect to a control measurement; LCC, lauroylcholine chloride; M, proposed manganese-containing charge-accumulating intermediate; P-680, reaction center chlorophyll *a* molecule; Phe, reaction center pheophytin molecule; PQ, plastoquinone; PS, Photosystem; Q_A, Q_B, primary and secondary quinone acceptors; S-states, the S_n ($n = 0, 1, 2, 3, 4$) states of Photosystem II as governed by the oxidation level of M; Z, first electron donor to P-680; Tris, 2-amino-2-hydroxymethylpropane-1,3-diol.

Introduction

The photosynthetic water-splitting process in higher plants is mediated by Photosystem II (PS II), a chlorophyll (Chl) -containing protein complex embedded in the thylakoid membrane matrix of the chloroplast. The overall process involves the photooxidation of a specialized reaction center Chl *a* molecule, P-680, which extracts electrons from water via at least two intermediates and causes the reduction of quinone-containing components that are utilized by Photosystem I (PS I) in the photoreduction of NADP. Although the kinetics of the reactions are well characterized, the exact chemical nature of the water-splitting process itself remains unknown (for reviews, see Refs. 1–3). From O_2 flash yield kinetic measurements, it has been postulated that a special intermediate (assumed to be a manganese-containing component polypeptide) is sequentially oxidized upon four successive photoexcitations of P-680 before it reacts to cause the release of O_2 and begins the cycle over again. This so-called charge-accumulating intermediate accounts for the S-state transitions in PS II [4,5] and is defined as 'M' in this paper. Likewise, based on proton flash yield kinetic measurements of the protons released from the oxidizing side of PS II in the first few light flashes [6–12], the stepwise oxidation of M is assumed to be tighly coupled to the partial oxidation of bound water molecules [1–3]. However, again the nature of the partially oxidized water species is unknown. These two fundamental observations form the underlying experimental basis for the stepwise oxidation models, usually involving manganese, for photosynthetic water splitting currently in vogue in the literature [13–19].

Recently, we have introduced the use of choline/fatty acid-derived compounds, in particular lauroylcholine chloride (LCC), to specifically probe PS II structure and function [20]. Based on fluorescence yield transient measurements we have shown that, depending upon the concentration, LCC can be used to block individual PS II reaction steps sequentially [21]. In this paper we report our findings at relatively low LCC concentrations in thylakoid samples. Under this condition no detectable O_2 evolution or uptake can be measured either under continuous or flashing light regimes; however, steady-state electron flow through PS II and the apparent normal cycling of the M intermediate (i.e., the S-state transitions) as monitored by the microsecond range Chl *a* delayed fluorescence still occurs. LCC does not appear to interfere with the O_2 yield measurements directly nor act as an electron donor itself under the conditions in which the O_2 evolving capacity is lost. Although the full significance of these results is still uncertain at this time, the data are interpreted in terms of the possible existence of an additional water binding component (termed 'H') in PS II and a concerted reaction mechanism for photosynthetic water splitting, as opposed to the stepwise oxidation models as mentioned above. Some of these results were reported at the 9th International Congress on Photobiology, Philadelphia, PA, July 1–6, 1984.

Experimental

Sample preparation and treatment with lauroylcholine chloride

Thylakoid membrane samples were prepared either from greenhouse-grown peas or market spinach according to the procedure of Robinson and Yocum [22]. The samples were treated with 2 mM EDTA and then washed twice in the suspension buffer medium to remove the EDTA. Tris inactivation of O_2 evolution was performed under the usual conditions of 0.8 M Tris (pH 8.2) for 20 min in dim light on ice. Tris-treated samples were then centrifuged and resuspended in the suspension buffer medium. For these experiments it did not seem to matter whether fresh or frozen ($-70°C$) samples were used, although the best activities were obtained with the fresh samples and these were used for most of the experiments.

Samples were treated with lauroylcholine chloride (LCC) in the amounts indicated in the figures. Reagent grade material was obtained either from US Biochemical Corp. or Sigma Chemical Co. The Sigma brand was found to have an over 95% purity as determined by melting point and mass spectrometry analysis. Further purification by simple recrystallization from saturated ethanol solutions proved to be difficult. As LCC may degrade at room temperatures, fresh stock solutions were prepared daily in 100% ethanol and aliquots were

added to the thylakoid samples with rapid mixing to give a final ethanol concentration of not more than 2%. All controls contained comparable amounts of ethanol. LCC was added in a ratio to the Chl content of the sample (i.e., LCC : Chl, w/w). At the relatively low LCC amounts used in these experiments, the LCC effect was maximal within 3 min incubation time, with little further effect up to 10 min. Minimum 3 min incubation times were therefore used.

Activity measurements

Steady-state O_2 evolution and uptake measurements were made with a YSI Model 51 Clark electrode set-up using a 1.5 ml Gilson Model OX15253 water jacketed cell. The temperature during the measurements was maintained at 24°C with a controlled temperature water circulator. Saturating actinic light was provided by an American Optical Illuminator Model 755 (200 W lamp) and was focused through an Oriel LP.47 yellow filter and a Corning CS 1-75 heat filter. The assay conditions for the O_2 evolution measurements were: 0.4 M sucrose, 50 mM Hepes, 10 mM NaCl and 5 mM $MgCl_2$ standard buffer medium (pH 7.5), 3 mM $K_3Fe(CN)_6$ and 0.35 mM 2,6-dimethyl-*p*-benzoquinone. For the PS II donor reaction measured as O_2 uptake, the assay conditions were: Tris-inhibited samples, standard buffer medium (pH 7.5), 0.4 mM NaCN, 0.1 mM methyl viologen and either 5 mM NH_2OH or 0.25 mM phenylenediamine/2 mM ascorbate as the electron donor. For the PS I donor reaction measured as O_2 uptake, the assay conditions were: standard buffer medium (pH 7.5) 0.4 mM NaCN, 0.1 mM methyl viologen, 3.3 μM 3-(3',4')dichlorophenyl)-1,1-dimethylurea (DCMU) and either 0.05 mM 2,6-dichlrorophenolindophenol (DCIP)/2 mM ascorbate or 0.5 mM durohydroquinone/$NaBH_4$. The O_2 uptake reactions in the presence of ascorbate were checked and subsequently corrected for superoxide production by using excess superoxide dismutase [23]. As an uncoupler, 2 μM gramicidin D was added to all assay mixtures. The sample concentration was 30 μg Chl/ml.

Steady-state ferricyanide photoreduction was measured at 420 nm using a Cary 17D dual beam spectrophotometer. A Corning CS 4-96 blue filter and a 5429- 4 narrow bandpass 420 nm filter were placed in front of the photodector. Saturating actinic light was obtained from an Ealing quartz/halogen 150 W fiber optic light source and was passed through a Corning HR 90 heat reflecting filter and two Corning CS 7-58 red filters. The assay conditions were the same as for the steady-state O_2 evolution measurements except 10 μg Chl/ml was used.

Oxygen flash yield measurements were made on a laboraotry built bare platinum electrode [24]. Saturating white light flashes were obtained from a General Radio Strobotac Model 1538-A xenon flash lamp with a high energy output capacitor. Light flashes with full width at half maximum (FWHH) 3 μs were used at a 1 Hz repetition rate. Time response of the electrode was approx. 2 ms. The assay conditions were: standard buffer medium (pH 7.5), 2 μM gramicidin D and 500 μg Chl/ml. The experimental protocol consisted of 40 preilluminating flashes, 5 min dark period, and then the O_2 flash yield measurement.

The seconds range Chl *a* fluorescence transients at 685 nm were measured on a SLM Model 4800 spectrofluorimeter modified for transient measurements. Monochromatic actinic illumination was at 440 nm. The assay conditions were the same as for the O_2 flash yield measurements except 5 μg Chl/ml was used. The experimental protocol consisted of 10 s preilluminating light, 5 min dark period, and then the transient measurement.

The microsecond range Chl *a* fluorescence yield at 685 nm was measured on a laboratory built kinetic spectrofluorimeter [25]. The assay conditions were the same as for the fluorescence transient measurements. The experimental protocol consisted of subjecting a preilluminated, 3 min dark-adapted sample to a train of flash pairs at a repetition rate of 10 Hz. Each flash pair consisted of a saturating flash followed 80 μs later by a weak (non-photoactivating) flash. The FWHH of the flashes was 3 μs. The fluorescence yield was monitored simultaneous with the second flash in the flash pair. A detailed discussion of this technique has been given earlier [26].

The microsecond range Chl *a* delayed fluorescence measurements were made on the same kinetic spectrofluorimeter [25]. The assay conditions were the same as for the fluorescence transient measurements. The experimental protocol consisted of

subjecting a preilluminated, 3 min dark-adapted sample to a train of single saturating light flashes at a repetition rate of 1 Hz. The photomultiplier was switched off during the excitation flashes with an electronic gating circuit (cut off, approx. $5 \cdot 10^3$) and then turned on at 130 μs after each flash, which allowed delayed fluorescence at 140 μs or longer to be measured.

Results

Fig. 1 shows the dependence of steady-state photosynthetic activity measurements of thylakoid samples upon LCC concentration. Note that the amount of LCC to cause 50% inhibition with respect to the control (I_{50}) is about LCC : Chl = 2.5 : 1 for O_2 evolution and about LCC : Chl = 5 : 1 for the PS II donor reaction. The PS I donor reaction, on the other hand, is uninhibited up to LCC : Chl = 10 : 1. These results are consistent with our earlier findings that LCC can be used to inhibit selectively PS II reactions [21].

The PS I donor reaction shown in Fig. 1 is significantly enhanced by LCC, with a maximum increase at about LCC : Chl = 5 : 1. This increase is not due to an uncoupling effect, since gramicidin D was included in all assays as an uncoupler.

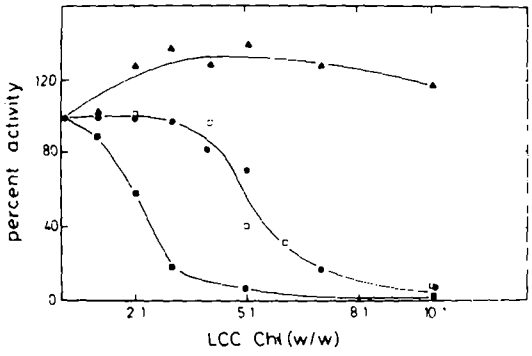

Fig. 1. Plot of percent activity with respect to the control as a function of the amount of LCC (in a ratio with Chl) in thylakoid samples for: (1) O_2 evolution (■); (2) ferricyanide photoreduction (●); (3) the PS II donor reaction in Tris-inhibited samples (□); and (4) the PS I donor reaction in the presence of DCMU (▲). The control rates with ±7% S.D. for the various reactions were: 840 μequiv./mg Chl per h, 803 μequiv./mg Chl per h, 298 μequiv./mg Chl per h, and 554 μequiv./mg Chl per hr, respectively. Assay conditions are given in the Experimental section.

Rather, it appears to be related to an increase in the P-700 signal amplitude (i.e., the reaction center Chl *a* molecule of PS I) that we reported earlier (see Table III of Ref. 20).

The striking feature in Fig. 1 is the ferricyanide photoreduction curve. The I_{50} in this case is about LCC : Chl = 5 : 1. The loss in ferricyanide photoreduction with increasing LCC concentrations parallels the loss in the PS II donor reaction rather than in O_2 evolution, although no electron donors were added to the samples during the measurement. The samples and assay conditions were identical for both the O_2 and ferricyanide photoreduction measurements (the amount of LCC added was, of course, adjusted to the Chl concentration to give the appropriate ratios). The partially inhibited rates in both measurements at the low LCC amounts were linear up to about 1 min illumination time. These results thus indicate that, for example, at LCC : Chl = 5 : 1 PS II can operate at more than 50% capacity as monitored by ferricyanide photoreduction when there is virtually no detectable O_2 evolution in these samples. We have obtained similar results with Triton X-100 prepared PS II sub-membrane fractions for O_2 evolution and DCIP photoreduction (see Fig. 1 of Ref. 27). However, the I_{50} values may vary with different sample preparations and, as we will show in a later paper, depend upon experimental conditions including pH, salt concentration, osmoticum, and the presence of Ca^{2+} ions, polyethyleneglycol or trace, non-dissociating amounts of other detergents such as digitonin.

There is a number of possible trivial explanations for our observation that electron flow continues even though no O_2 evolution can be detected. First, LCC may interfere with the electrode's ability to measure O_2. This appears not to be the case, since O_2 levels as measured by the Clark electrode are unaffected by LCC in the presence or absence of light (e.g., see the O_2 traces in Fig. 1 of Ref. 20). Second, free peroxide or superoxide may perhaps be generated during the reactions in the light. However, this also seems unlikely, since O_2 levels are unaffected with LCC inhibited samples in the light when either peroxidase, catalase or superoxide dismutase is added to the assay mixture (data not shown).

A third possibility is that LCC may induce high

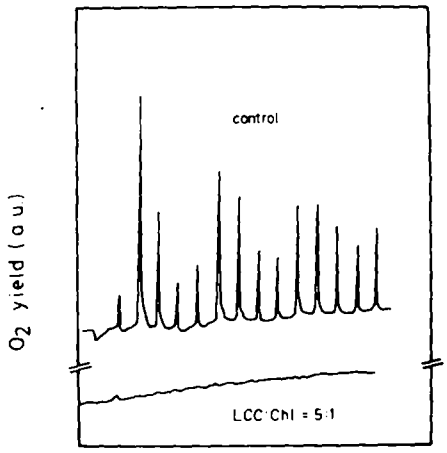

Fig. 2. O_2 flash yield measurements of thylakoid samples with and without LCC:Chl = 5:1. Time resolution of O_2 detection was approx. 2 ms. Assay conditions are given in the Experimental section.

levels of transient O_2 consumption. To determine this we measured the O_2 flash yields of the samples. Fig. 2 shows the O_2 traces from O_2 flash yield measurements in the absence of electron acceptors for control thylakoid samples and thylakoid samples inhibited with LCC:Chl = 5:1. The control samples show the normal O_2 flash yield pattern [28]. However, there is no net detectable O_2 evolution or uptake in the presence of the LCC, although according to Fig. 1 PS II is operating at more than 50% capacity under this condition. These results clearly show that transient O_2 uptake, at least within the 2 ms time response of the electrode, does not compensate for any O_2 that may be produced in LCC treated samples. We obtained similar results for Triton X-100 prepared PS II sub-membrane fractions (see Fig. 2 of Ref. 27).

The critical micelle concentration for alkyl quarternary amine detergents is in the range of 1–20 mM, depending of course upon the ionic strength and other experimental parameters [29]. Except for the O_2 flash yield measurements, all assays were measured with LCC concentrations below 1 mM (for a sample containing 30 μg Chl/ml at LCC:Chl = 5:1, the LCC concentration is 0.46 mM) and therefore probably below the critical micelle concentration. But for the O_2 flash yield measurements reported in Fig. 2, the LCC concentration was 7.6 mM which could be within the critical micelle concentration range under the experimental conditions employed. To determine whether there were any dramatic LCC concentration effects on the inhibition curve for O_2 evolution, we treated samples at 500 μg Chl/ml with LCC and then diluted the sample to 30 μg Chl/ml for O_2 evolution measurements as in Fig. 1. We found the I_{50} in this case to be about LCC:Chl = 1.5:1 (data not shown), not dramatically different from what is shown in Fig. 1. We believe that the differential effects we observe in the photosynthetic activity measurements in Fig. 1 arise from subtle interactions between LCC and the polypeptide components within the membrane. However, concentration effects and micelle formation may very well be important in the dissociation and solubilization of the membrane components by LCC.

Another possible explanation for the behavior of the O_2 evolution and ferricyanide photoreduction curves in Fig. 1 is that LCC itself acts as an electron donor. This is somewhat unlikely in that lauroyl choline is a cationic species, i.e.,

$$(CH_3)_3\overset{+}{N}CH_2CH_2O\overset{O}{\overset{\|}{C}}(CH_2)_{10}CH_3$$

and a priori would not be expected to donate electrons. However, in order to determine whether LCC may act as an electron donor, we measured the seconds range Chl *a* fluorescence transient in control and Tris inhibited thylakoid samples with and without LCC.

The variable component of the fluorescence transient (ΔF), defined as the difference between the instantaneous fluorescence level (F_0) and the maximum fluorescence level (F_{max}), reflects the conversion of the primary quinone acceptor in PS II (Q_A) from a quenching state to a nonquenching state [30]. This occurs when electron flow to Q_A from the PS II reaction center is more rapid than electron flow away from Q_A to the intersystem plastoquinone (PQ) pool and PS I [31]. Fig. 3a shows the fluorescence transients for control thylakoid samples and for thylakoid samples treated with LCC:Chl = 5:1. Note that the ΔF is virtually unaffected by the presence of the LCC, indicating that sufficient electron flow occurs

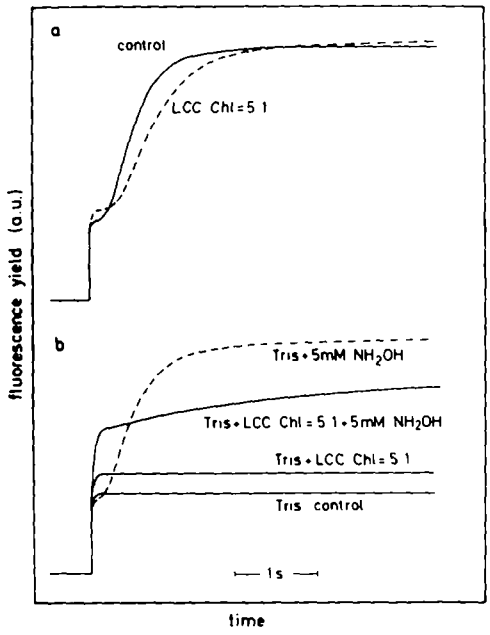

Fig. 3. Chlorophyll fluorescence yield transient measurements with and without LCC:Chl = 5:1 for: (a) thylakoid samples and (b) Tris-inhibited thylakoid samples. The top traces in (b) are for Tris-inhibited samples containing 5 mM NH_2OH as a PS II electron donor. Assay conditions are given in the Experimental section.

through PS II to fully reduce Q_A and the intersystem PQ pool, even though from the results in Figs. 1 and 2 there is no detectable O_2 evolution under this LCC condition. There is, however, a small (approx. 17%) increase in the apparent F_0 level in the LCC-treated samples compared with the control samples. We have reported similar observations earlier (see Table IV of Ref. 20 and Fig. 1 of Ref. 21). This increase in the apparent F_0 level may be related to the perturbation of the PS II core chlorophyll light-harvesting complex by LCC. We have found, for example, that LCC can cause the release of the chlorophyll complexes which give rise to the 695 nm emission band at 77 K (e.g., see Fig. 2 of Ref. 20). In addition, the kinetics of the fluorescence rise is somewhat slower in the presence of the LCC. This may be due to a decreased quantum transfer efficiency to P-680 if the core light-harvesting complex becomes disconnected from the reaction center under this LCC condition. Alternatively, LCC may somehow cause an increase in the reduction capacity of the intersystem PQ pool. Fig. 3b shows the fluorescence transients for Tris-inhibited thylakoid samples. Tris treatment of thylakoid samples is known to inhibit the oxidizing side of PS II by blocking electron flow from water to P-680 [1]. This results in a low fluorescence level because Q_A cannot be reduced by PS II as rapidly as it is oxidized by PS I. This is shown by the bottom trace in Fig. 3b. However, when an exogenous electron donor to PS II is added to a Tris-inhibited sample, such as NH_2OH, F_{max} increases and ΔF is restored as Q_A is now more rapidly reduced that it is oxidized. This is shown by the top trace in Fig. 3b. When LCC is added to the Tris-inhibited sample at LCC:Chl = 5:1, the fluorescence level remains low, but increases upon the addition of NH_2OH, indicating restoration of ΔF and electron flow to Q_A. These effects are shown by the two middle traces in Fig. 3b. For the Tris-inhibited sample containing only LCC, the fluorescence level is about 25% higher compared with the Tris inhibited control. We interpret this as arising, at least in part, from the increase in the apparent F_0 level which is induced by LCC (see above). For the Tris-inhibited sample containing both LCC and NH_2OH, the F_{max} attains a level within 4 s to about 80% of the F_{max} in the Tris-inhibited sample containing only NH_2OH. However, the kinetics are dramatically altered, showing a rapid initial increase and then a much slower rise to F_{max}. In essense, this behavior in the kinetics is somewhat similar to what is shown in Fig. 3a for a thylakoid sample treated with LCC only, except that the effects are considerably enhanced. These results may indicate that there is a synergistic interaction between Tris treatment and LCC treatment, but further experiments are needed to clarify the nature of this interaction. Nevertheless, we conclude from these results that LCC does not act as an efficient electron donor to PS II after the Tris block. In addition, we note that in the PS II donor measurements reported in Fig. 1, no detectable electron flow can be measured in the presence of low LCC amounts without the addition of NH_2OH or reduced phenylene diamine as electron donors. We have reported similar fluorescence results using Triton X-100 prepared PS II submembrane fractions (see Fig. 3 of Ref. 27).

The other possible explanation to account for the behavior of the O_2 evolution and ferricyanide photoreduction curves in Fig. 1 is that LCC in

some way interferes with the ferricyanide photoreduction reaction, although throughout our experience we have obtained similar results with LCC using a variety of exogenous electron acceptors including DCIP, variously substituted *p*-benzoquinones and methyl viologen. The results in Fig. 3a do indicate that in the presence of LCC : Chl = 5 : 1, sufficient electron flow can occur through PS II to reduce Q_A in the absence of exogenous electron acceptors. However, the fluorescence transient measurements require only a few turnovers of PS II to reduce Q_A and the intersystem PQ pool. Thus, we still wanted to determine whether large numbers of PS II turnovers can occur in the presence of LCC without the complication of possible interference between LCC and added exogenous electron acceptors. This was accomplished by measuring the microsecond range Chl *a* fluorescence under multiple flash conditions. For these experiments thylakoid samples were subjected to a train of double flash pairs. The first saturating flash in the pair induces a single charge separation at the PS II reaction center, while the second weak flash monitors the fluorescence yield, and hence the reduction level of Q_A. The time between the strong and weak flashes was 80 μs which is sufficiently short to be within the lifetime of Q_A^-, while the repetition rate of the flash pairs at 10 Hz allows sufficient time for Q_A^- to recover largely to its ground state between each flash pair in the train [32]. The results are shown in Fig. 4 which is a plot of the normalized variable fluorescence ($\Delta F/F_0$) at 80 μs as a function of the number of flash pairs. For control thylakoid samples the fluorescence increases on the first flash pair and stays high on succeeding flash pairs out to 200 flash pairs (top plot, Fig. 4a). We do not observe a binary oscillation in the fluorescence yield for our controls as reported earlier [33] because of the time frame used in our measurements. In any event, PS II appears to operate normally and Q_A becomes reduced with each turnover of PS II. For thylakoid samples treated with LCC : Chl = 5 : 1, the fluorescence also increases on the first flash pair and stays high out to 200 flash pairs (bottom plot, Fig. 4a) as in the control, even though according to Figs. 1 and 2 this sample does not produce detectable O_2 and no exogenous electron donors were added. For comparison, Fig. 4b

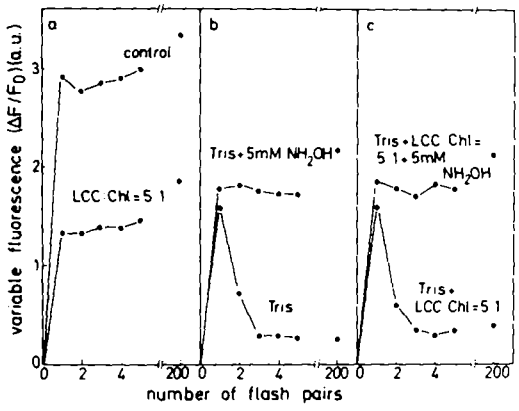

Fig. 4. Photosystem II turnover measurements as determined by the flash-induced chlorophyll fluorescence yield at 80 μs as a function of the number of double flash pairs for: (a) thylakoid samples with and without LCC : Chl = 5 : 1; (b) Tris-inhibited thylakoid samples with and without 5 mM NH_2OH; and (c) Tris inhibited thylakoid samples containing LCC : Chl = 5 : 1 with and without 5 mM NH_2OH. Assay conditions are given in the Experimental section.

shows the PS II turnover measurements for Tris-inhibited samples which are characteristic for other treatments known to inhibit O_2 evolution by blocking electron flow on the oxidizing side of PS II [26]. The first flash pair induces a high fluorescence level, indicating that at least one electron can be transfered through the PS II reaction center to reduce Q_A. But on succeeding flash pairs the fluorescence drops and remains low as this electron donor pool is depleted and cannot be rereduced within the time of the flash pair repetition rate. When 5 mM NH_2OH is added as an exogenous electron donor to the Tris inhibited sample, the fluorescence increases and stays high out to 200 flash pairs, indicating sequential electron transfer to Q_A (top plot, Fig. 4b). Finally, Fig. 4c shows the results from Tris-inhibited samples containing LCC : Chl = 5 : 1 with and without 5 mM NH_2OH. The behavior of the flash patterns in this case is similar to those in Fig. 4b for Tris-inhibited samples without LCC, indicating that in the LCC-treated samples electron transfer to Q_A remains operational.

The results in Fig. 4 parallel the behavior in ΔF obtained from the transient measurements in Fig. 3. However, for both Tris inhibited plus NH_2OH and LCC treated samples, there is a considerable

quenching of the ΔF measured in the microsecond time range as compared with the ΔF measured in the seconds time range, more than can be accounted by the increase in the apparent F_0 level. It is known that the fluorescence yield dynamically varies over this time span depending upon the kinetics of the electron transfer events and quenching states generated in PS II [34]. Although both Tris and LCC do not block electron flow through PS II from exogenous electron donors such as NH_2OH, these treatments may, however, alter the kinetics of the electron transfer reactions in a way to affect variously the ΔF in different time frames. However, to quantitate these effects will require further investigation. For the present purpose of this paper, we conclude from the results shown in Fig. 4 that at LCC : Chl = 5 : 1 normal electron flow can occur through those PS II centers that remain operational for a minimum of 200 turnovers without detectable O_2 evolution in the absence of added electron donors and acceptors.

Since under the conditions of LCC : Chl = 5 :'1 a large fraction of PS II centers appears to operate normally without detectable O_2 evolution, we wanted to know whether the S-state transitions still occur. To get information on this, we measured the microsecond range Chl *a* delayed fluorescence as a function of saturating light flashes. Although the source of delayed fluorescence is uncertain [32,35] and may arise from damaged PS II centers inactive in O_2 evolution [36], the microsecond range delayed fluorescence does appear to be modulated by the S-state transitions [36,37]. The results in Fig. 5a shows a plot of the relative amplitude of the delayed fluorescence at 150 μs for control thylakoid samples and thylakoid samples treated with LCC : Chl = 5 : 1 as a function of flash number. As with the control, the LCC-treated sample shows oscillations with period four and peaks after the 3rd and 7th flashes, although the LCC-treated sample does not produce detectable O_2 (Fig. 2). Fig. 5b shows the oscillations for the two types of samples normalized to the same value at the 5th flash. Note that the extent of the damping in the oscillations is about the same for both samples, indicating that the apparent S-state cycling is the same in both samples.

According to the interpretation of Buttner and Babcock [36], in uninhibited thylakoid samples the

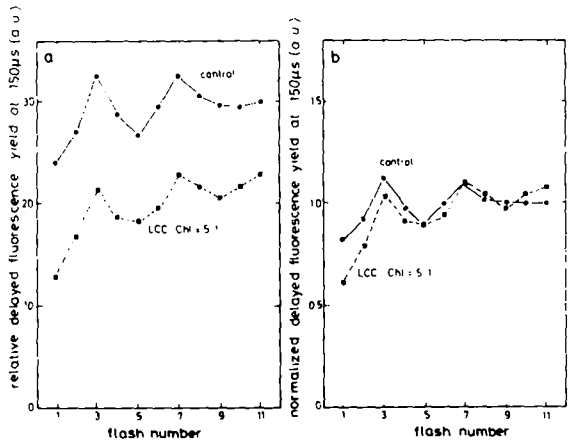

Fig. 5. Plot of the flash-induced chlorophyll-delayed fluorescence amplitude at 150 μs as a function of flash number for thylakoid samples with and without LCC : Chl = 5 : 1. (a) Plot of the relative delayed fluorescence yield. (b) Plot of the delayed fluorescence Yield normalized at the 5th flash. Incubation time with the LCC was 5 min. Assay conditions are given in the Experimental section.

microsecond range delayed fluorescence arises from P-680$^+$Q$_A^-$ charge recombination in the fraction of PS II centers inactive in O_2 evolution and is modulated in amplitude with period four oscillations upon flash excitation by the local charge densities generated at the active O_2-evolving sites in the sample. They note that the relationship between O_2 activity and the oscillatory behavior is not strictly linear, that it is possible to inhibit up to 35% of the O_2 activity without significantly altering the extent of the oscillations. But it is clear in their results that with total inhibition of O_2 evolution either by Tris treatment or gentle heating the oscillations are lost. In our case at LCC : Chl = 5 : 1, more than 95% of the O_2 activity is lost (see Figs. 1 and 2, where the $S/N \approx 100$) while the extent of the delayed fluorescence oscillations is near normal under the same LCC condition (Fig. 5b). However, the amplitude of the microsecond delayed fluorescence in the LCC treated sample as shown in Fig. 5a is about 65% of the control. This, interestingly, closely corresponds to the PS II capacity for electron flow under the same LCC condition as measured by ferricyanide photoreduction (Fig. 1). We find that the loss of the PS II capacity for ferricyanide photoreduction parallels a loss in the delayed fluorescence ampli-

tude. For example, at LCC : Chl = 10 : 1 where the ferricyanide photoreduction and PS II donor reaction is totally inhibited and the PS I donor reaction is comparable with the control, the 140 μs and longer delayed fluorescence is completely lost. This is shown in Fig. 6. Thus, LCC inhibition is quite different from This inhibition in which the total microsecond range delayed fluorescence amplitude remains at a level similar to the control. Buttner and Babcock [36] explain this lack of amplitude difference between uninhibited and Tris-inhibited samples as an exciton quenching effect of P-680$^+$ in the Tris-inhibited samples. The loss in amplitude upon LCC treatment may in part be possibly explained by this mechanism, but not the complete loss. Alternatively, we may a priori suggest that the loss in amplitude may be due to a block in charge separation at the reaction center by LCC. But we note that the dithionite-induced increase in the Chl *a* fluorescence in Tris-inhibited samples, which reflects the integrity of Q_A (and hence, presumably, the integrity of the reaction center complex), is prevented by LCC only at very high concentrations, the I_{50} being about LCC : Chl = 28 : 1 (see Fig. 4 of Ref. 21). Another possibility for the loss in amplitude is that LCC affects the emission yield parameter. Further work is needed to clarify these possibilities.

Nevertheless, although we can not quantitate the number of PS II centers undergoing S-state transitions by our current delayed fluorescence measurements, we conclude that a significant fraction of the PS II centers can undergo S-state transitions in the absence of detectable O_2 evolution, and presumably water-splitting activity, upon treatment of thylakoid samples with LCC : Chl = 5 : 1.

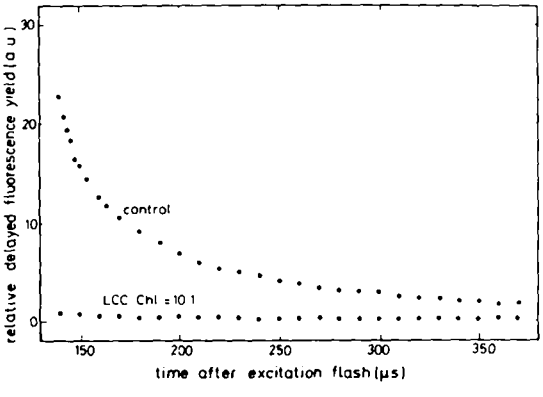

Fig. 6. Plot of the 140–370 μs chlorophyll-delayed fluorescence for thylakoid samples with and without LCC : Chl = 10 : 1. Assay conditions are given in the Experimental section.

Discussion

In our earlier work we have employed choline/fatty acid-derived compounds, in particular lauroylcholine chloride (LCC), to inhibit selectively PS II reactions, and to exclude core PS II polypeptides from the membrane depending upon the concentration [20,21,27]. In this paper we show that by using relatively low amounts of LCC, detectable O_2 evolution can be uncoupled from normal PS II electron flow and apparent cycling of the S-state transitions in a significant fraction of the PS II centers in thylakoid membrane samples. Fig. 1 shows that the I_{50} for O_2 evolution is considerably less than it is for ferricyanide photoreduction under identical assay conditions (the I_{50} is about LCC : Chl = 2.5 : 1 and LCC : Chl = 5 : 1, respectively). At the concentration of LCC : Chl = 5 : 1 no detectable O_2 evolution can be measured, yet more than 50% of the PS II centers appears to operate normally in terms of electron flow without added electron donors. The LCC at this concentration does not appear to interfere with the O_2 yield and ferricyanide photoreduction measurements directly (see arguments related to Figs. 1–4). LCC, for example, does not cause transient O_2 uptake within a 2 ms response time (Fig. 2). Also LCC at this concentration does not appear to act as an electron donor itself to PS II between the Tris block and the P-680 (Fig. 3), although a minimum of 200 turnovers of PS II can occur without the addition of electron donors (Fig. 4). In addition, LCC at the concentration of LCC : Chl = 5 : 1 where it inhibits O_2 evolution detectable by both steady state and flash yield measurements (Figs. 1 and 2) still allows apparent normal cycling of the S-state transitions in those centers which are still operational in terms of electron flow (more than 50% of the control, Fig. 1), as monitored by the period four oscillations in the microsecond range delayed fluorescence (Fig. 5).

We feel that these results – the uncoupling of detectable O_2 evolution from normal electron flow

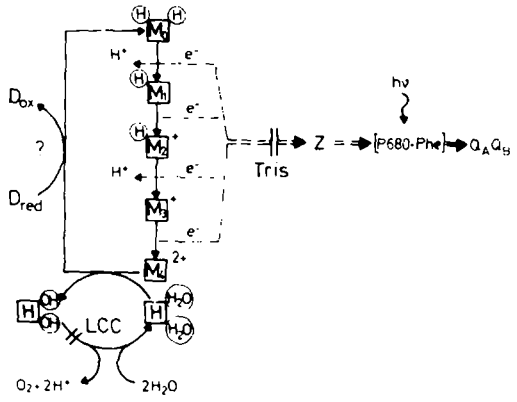

Fig. 7. Concerted reaction model for photosynthetic water splitting. 'H' is a theoretical water-binding component. Explanation of the symbols and details are given in the text.

through PS II without the addition of electron donors – may have important implications in the water-splitting process. Many of the current models for water splitting entail a stepwise process involving the partial oxidation of bound water molecules [13–19]. However, our current results suggest that this may not necessarily be the case. Although we do not understand the full significance at this time, one possible explanation is that water-splitting occurs via a concerted reaction mechanism, as originally implied in the S-state model of Kok and co-workers [4,5]. Fig. 7 presents a model in which water splitting is separated from the charge accumulating intermediate in PS II. The symbols in Fig. 7 have their usual meaning with P-680 being the PS II reaction center Chl *a* molecule; Phe, the primary pheophytin electron acceptor; Q_A and Q_B, the primary and secondary quinone electron acceptors, respectively; Z, the first electron donor to P-680; M, the manganese-containing charge-accumulating intermediate which accounts for the S-state transitions in PS II; and H, a theoretical water-binding component, which we propose reacts with M in a concerted reaction. To account for our results, therefore, we suggest that LCC, at the relatively low amounts used in these experiments, blocks water splitting at the component H, thus allowing M to cycle and the rest of PS II to operate normally.

The important question remains, however, as to what is the source of electrons under this condition. Although LCC or possible contaminating species do not appear to donate electrons to PS II after the Tris block (Fig. 3), they may donate electrons directly to M. But at this point we note that based on the purity of the Sigma LCC (see the Experimental section) and the amounts used in the photosynthetic assay measurements of Fig. 1, at most about 350 contaminating molecules per PS II unit may be present. This amount of possible contaminants could not explain the electron source to sustain the prolonged (more than 1 min) steady-state ferricyanide reduction rates that are observed in the absence of detectable O_2 evolution, unless some catalytic or cyclic redox reaction mechanism is involved. Alternatively, a large endogenous electron donor pool may exist, or general photooxidation reactions with Chl or intrinsic membrane components could be the electron source. Here again we note that the partially inhibited ferricyanide photoreduction rates at the low LCC amounts are fairly linear within a 1 min illumination time. We have not as yet measured photodegradation reactions under LCC conditions. Still another possibility is that a back flow of electrons from the quinone acceptor intermediates (Q_A or Q_B) is favored in the presence of LCC when no O_2 evolution is detectable, but this mechanism would be difficult to explain the relatively high ferricyanide photoreduction rates which would be in competition for the electrons under this condition. In all of these explanations, electron donation would have to be mediated through the M_4 state to allow the normal cycling of the S-state transitions that we observe. These possible electron donor reactions are represented by the reaction $D_{red} \rightarrow D_{ox}$ in Fig. 7. A final possibility is that LCC at the low concentrations interferes somehow with the water-splitting chemistry such that the final dioxygen product is not produced or not released. But we note that free peroxide does not appear to be formed. Further experiments are needed to clarify what is the source of electrons under these experimental conditions.

To account for the flash proton release patterns in dark-adapted samples that have been reported in the literature [6–12], the model in Fig. 7 must assume that the first protons released in flashing light do not come directly from water, but rather from some proton groups located on the M intermediate. These proton groups are later replenished

by water protons after the first cycle of the M intermediate, so that all protons ultimately come from water. In support of this idea, there is some evidence in the literature that protons released during water oxidation do not initially enter the intrathylakoid compartment [38]. Also, it has been observed [10,12] that hours of dark adaptation are needed to obtain the 'normal' 1,0,1,2-proton-release pattern, while only 10 min are needed to obtain the normal O_2 pattern [28]. Crucial to the model in Fig. 7 would be proton flash yield measurements under the LCC condition where there is no detectable O_2 evolution, yet substantial electron flow through PS II. We are attempting to undertake these measurements.

In conclusion, in this paper we have reported an unusual condition at relatively low amounts of LCC in thylakoid samples in which detectable O_2 evolution can be uncoupled from electron flow and the apparent S-state cycling in PS II. Although we do not fully understand the significance of the results at this time, we feel that this observation may have important implications in the photosynthetic water-splitting mechanism and have attempted to explain the results in terms of a hypothetical water binding component and a concerted reaction mechanism in PS II. Further work now in progress on the effects of LCC on PS II biochemistry and charge transfer reactions should help us evaluate the nature of this phenomenon.

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References

1 Wydrzynski, T. (1982) in Photosynthesis – Energy Conversion by Plants and Bacteria, Vol. I (Govindjee, ed.), pp. 469–506, Academic Press, New York
2 Amesz, J. (1983) Biochim. Biophys. Acta 726, 1–12
3 Renger, G. and Govindjee (1985) Photosynthesis Res. 6, 33–55
4 Kok, B., Forbush, B. and McGloin, M. (1970) Photochem. Photobiol. 11, 457–475
5 Forbush, B., Kok, B. and McGloin, B. (1971) Photochem. Photobiol. 14, 307–321
6 Fowler, C.F. (1977) Biochim. Biophys. Acta 462, 414–421
7 Junge, W., Renger, G. and Ausländer, W. (1977) FEBS Lett. 79, 155–159
8 Saphon, S. and Crofts, A.R. (1977) Z. Naturforsch. Biosci. 32C, 617–626
9 Velthuys, B.R. (1980) FEBS Lett. 115, 167–170
10 Förster, V., Hong, Y.-Q. and Junge, W. (1981) Biochim. Biophys. Acta 638, 141–152
11 Wille, B. and Lavergne, J. (1982) Photobiochem. Photobiophys. 4, 131–144
12 Förster, V. (1984) Ph.D. Thesis, Universität Osnabrück, Germany
13 Govindjee, Wydrzynski, T. and Marks, S.B. (1977) in Bioenergetics of Membranes (Packer, L. and Papageorgiou, G., eds.), pp. 305–316, Elsevier/North-Holland, Amsterdam
14 Renger, G. (1978) in Photosynthetic Water Splitting (Metzner, H., ed.), pp. 229–348, Academic Press, London
15 Wydrzynski, T. and Sauer, K. (1980) Biochim. Biophys. Acta 589, 56–70
16 Dismukes, G.C., Ferris, K. and Watnick, P. (1982) Photobiochem. Photobiophys. 3, 243–256
17 Andreasson, L.-E., Hansson, Ö. and Vänngard, T. (1983) Chimica Scripta 21, 71–74
18 Kusonoki, M. (1984) in Advances in Photosynthesis Research (Sybesma, C., ed.), Vol. I, pp. 275–278, Martinus Nijhoff/Dr. W. Junk Publishers, Dordrecht, The Netherlands
19 Goodin, D.B., Yachandra, V.K., Britt, R.D., Sauer, K. and Klein, M.P. (1984) Biochim. Biophys. Acta 767, 209–216
20 Wydrzynski, T. and Huggins, B.J. (1983) in The Oxygen Evolving System of Photosynthesis (Y. Inoue, A.R. Crofts, Govindjee, N. Murata, G. Renger and K. Satoh, eds.), pp. 265–272, Academic Press, Tokyo
21 Wydrzynski, T., Huggins, B.J. and Jursinic P.A. (1984) in Advances in Photosynthesis Research (Sybesma, C., ed.), Vol. I, pp. 437–440, Martinus Nihofff/Dr. W. Junk Publishers, Dordrecht, The Netherlands
22 Robinson, H.H. and Yocum, C.F. (1980) Biochim. Biophys. Acta 590, 97–106
23 Izawa, S. (1980) Methods Enzymol. 69, 413–434
24 Fork, D.C. (1972) Methods Enzymol. 24, 113–122
25 Jursinic, P.A. and Stemler, A. (1982) Biochim. Biophys. Acta 681, 419–428
26 Theg, S.M., Jursinic, P.A. and Homann, P.H. (1984) Biochim. Biophys. Acta 766, 636–646
27 Wydrzynski, T. and Nakatani, H.Y. (1984) in Current Topics in Plant Biochemistry and Physiology (Randall, D., ed.), p. 164, University of Missouri Press, Columbia, MO
28 Joliot, P., Barbieri, G. and Chabaud, R. (1969) Photochem. Photobiol. 10, 309–329
29 Helenius, A., McCaslin, D.R., Fries, E. and Tanford, C. (1979) Methods Enzymol. 56, 734–749
30 Duysens, L.N.M. and Sweers, H.E. (1963) in Studies on Microalgae and Photosynthetic Bacteria, Special Issue, Plant Cell Physiol., pp. 353–372

31 Papageorgiou, G. (1975) in Bioenergetics of Photosynthesis (Govindjee, ed.), pp. 319–371, Academic Press, New York
32 Govindjee and Jursinic, P.A. (1979) Photochem. Photobiol. Rev. 4, 125–205
33 Bowes, J.M. and Crofts, A.R. (1979) Biochim. Biophys. Acta 547, 336–346
34 Mauzerall, D. (1972) Proc. Natl. Acad. Sci. USA 69, 1358–1362
35 Amesz, J. and Van Gorkom, H.J. (1978) Annu. Rev. Plant Physiol. 29, 47–66
36 Buttner, W.J. and Babcock, G.T. (1984) Biochim. Biophys. Acta 767, 272–287
37 Zankel, K. (1971) Biochim. Biophys. Acta 245, 373–385
38 Sadewasser, D.A. and Dilley, R.A. (1978) Biochim. Biophys. Acta 501, 208–216