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The effect of diisothiocyanostilbene-2,2′-disulfonic acid (DIDS), trifluoperazine and lauroylcholinechloride on P-680$^+$ reduction and oxygen evolution

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Comparative measurements of flash-induced oxygen yield and P-680$^+$ reduction kinetics were carried out under repetitive flash excitation of Photosystem II (PS II) particles. In control samples with intact oxygen evolution capacity, 67% of P-680$^+$ was reduced in the nanosecond time-scale. Upon modification of PS II particle preparations with the inhibitors diisothiocyanostilbene-2,2′-disulfonic acid, trifluoperazine or lauroylcholinechloride, a state in Photosystem II is achieved in as much as 50% of the centers in which the normal nanosecond component in the P-680$^+$ reduction kinetics remains intact for at least 128 actinic flashes without giving rise to a detectable contribution to O_2 evolution. Arguments are presented to show that under these special inhibitory conditions, the rapid transfer of electrons from the primary electron donor, Z, to P-680$^+$ is not mediated by a normal ADRY-type mechanism nor through electron donation by the compounds themselves. The results imply that the site of inhibition of these compounds is near the O_2 release site in the water-splitting enzyme and that the amplitude of the nanosecond component of the P-680$^+$ reduction kinetics does not necessarily correlate with oxygen-evolution capacity even under repetitive flash-excitation conditions.

Introduction

Photosynthetic water oxidation occurs at the water-oxidizing enzyme system Y after the accumulation of four positive redox equivalents which are generated by consecutive photooxidations of the primary donor of Photosystem II, P-680, a special chlorophyll *a* complex (see Ref. 1 for a recent review). The electron transfer from Y to P-680$^+$ involves at least one further redox component Z [2]. The transient photooxidation of P-680 can be monitored by flash-induced absorption changes at 830 nm and 688 nm [3–5]. In untreated samples with intact O_2 evolution capacity the re-reduction of P-680$^+$ was shown to occur for the most part in the nanosecond time-scale with half-

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Abbreviations: ΔA_{830}, absorption change at 830 nm; ADRY, acceleration of the deactivation reactions of the water-oxidizing enzyme system Y; ANT 2p, 2-(3-chloro-4-trifluoromethyl)-anilino-3,5-dinitrothiophene; CHAPS, 3-[(3-cholciamidopropyl)dimethylammonio]propanesulfonate; Chl, chlorophyll; DIDS, diisothiocyanostilbene-2,2′-disulfonic acid; LCC, lauroylcholinechloride; Mes, 4-morpholineethanesulfonic acid; P-680, reaction center chlorophyll-*a* molecule of Photosystem II; PS II, Photosystem II; Q_A and Q_B, first and second quinone acceptors in Photosystem II; S_i, redox state of system Y; TFP, trifluoperazine; Z, primary electron donor to P-680.

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life times of 20–50 ns and 200–400 ns [3–5]. Under repetitive flash excitation, significant contributions of microsecond components to the P-680$^+$ reduction have been observed [5–8]. The origin of these microsecond components is still somewhat obscure.

Elimination of O_2-evolving capacity by treatments such as Tris-washing or incubation with NH_2OH is accompanied by an alteration of the P-680$^+$ reduction from the nanosecond to the microsecond time-scale [3,9–11] with half-life times dependent on the experimental conditions. In Tris-treated samples, for instance, after the first excitation following dark-adaptation one observes a relaxation kinetics of the 830 nm absorption changes with a pH-dependent half-life time of 2–40 μs due to the electron flow from Z to P-680$^+$ [11]. But in subsequent flashes, as Z remains oxidized, P-680$^+$ becomes reduced by back reaction with the reduced plastoquinone acceptor Q_A with a half-life time of 130 μs [10–12]. These results raise two questions: (a) does there exist a 1 : 1 correlation between the O_2-evolution capacity and the amplitude of the nanosecond phase of P-680$^+$ reduction; and (b) is every procedure that eliminates the O_2-evolution capacity necessarily accompanied by a retardation of the electron flow from Z to P-680$^+$.

In order to address the above questions, the average oxygen yield per flash and the relaxation kinetics of the 830 nm absorption changes were measured under comparable conditions in the presence of substances which selectively affect oxygen evolution. Diisothiocyanostilbene-2,2′-disulfonic acid (DIDS) and trifluoperazine (TFP) have previously been found to inhibit DCIP photoreduction in PS II preparations [13]. The inhibitory sites for these compounds were inferred to be located prior to the Tris- and NH_2OH-inhibitory sites [13]. Another approach to modify the structure and function of PS II is the use of choline fatty acid-derived compounds. In particular LCC has been found to lead to a selective inhibition of the major PS II charge-transfer reactions [14,15]. At relatively low concentrations LCC was inferred to interact with the primary O_2-evolving site itself [15,16], while at higher concentrations polypeptides related to PS II are released from the membrane (data in preparation).

In this communication we report the effects of DIDS, TFP and LCC treatment of PS II preparations on the P-680$^+$ reduction kinetics and the average oxygen yield per flash. The data show that a marked inhibition of the oxygen-evolving capacity can be achieved without concomitant loss of the nanosecond phase of P-680$^+$ reduction.

Materials and Methods

PS II particles with high oxygen-evolution capacity were prepared from market spinach as described in Ref. 17 with the modifications as in Ref. 18, except that the applied Triton X-100/chlorophyll ratio was 20 : 1. Addition of LCC to these particles enhanced the light-scattering properties to the extent of hampering the measurements of the absorption changes. Therefore, for the experiments with LCC, we used PS-II enriched submembrane fractions which were prepared according to the procedure of Seibert and coworkers as described in Ref. 19, except that 0.2% CHAPS was used for the second detergent treatment (Toyoshima, Y., personal communication). Even though there was some PS I contamination in these preparations, for the LCC experiments we resorted to the digitonin/CHAPS samples, since they gave the best optical properties.

The flash-induced O_2 yield was measured as described earlier [20]. Saturating light flashes (FWHM = 10 μs) were obtained from a xenon flash lamp at a repetition rate of 1.7 Hz. The yield of O_2 evolution was calculated from the integrated signals over 120 flashes.

The measurements of the 830 nm absorption changes with nanosecond time resolution were performed with a single-beam flash photometer similar to the one described in Ref. 3. The measuring light was provided by a laser diode (TXF 8300, AEG-Telefunken, $\lambda = 828$ nm). By means of a microscope objective lens, the beam was focused through the cuvette (4 cm pathlength) onto a 1 mm aperture which was located in front of the photodetector (Avalanche Photodiode RCA C30872). In order to suppress the flash-induced fluorescence artefact, the distance between the cuvette and the aperture was about 1 m. The photodetector was further protected against fluorescence by a 830 nm interference filter. The pho-

todiode was coupled via a 50 MHz amplifier (Pacific 2A50) to a Tektronix 7912 digitizer. 128 signals were averaged at a repetition rate of 1.7 Hz and stored on floppy disks. The measurements with microsecond time resolution were carried out with a different configuration. In this case the measuring beam was provided by a xenon arc lamp (Osram, XBO 150) and the wavelength was selected by a 830 nm interference filter. The optical pathlength of the sample was 1 cm. The photodetector was coupled to a 1 MHz amplifier (Tektronix, AM 502) and 32 signals were averaged. Photosynthesis was excited by non-saturating pulses from a Q-switched frequency doubled Nd : YAG laser (Spektrum GmbH, Berlin; $\lambda = 532$ nm; duration, 7 ns).

Fluorescence was monitored as described in Ref. 21. Assay conditions (a) for DIDS and TFP experiments: 20 mM Mes (pH = 6.0), 1 mM $K_3Fe(CN)_6$, 50 μM Chl, DIDS or TFP incubation time was 5 min with stirring; (b) for experiments with LCC, 10 mM Mes (pH = 6.5), 1 mM $K_3Fe(CN)_6$, 50 μM Chl and either 10 mM or 200 mM NaCl. Samples were treated with LCC as described in ref. 16 in a ratio with Chl (i.e., LCC/Chl (w/w)) as indicated in the figure legends. The incubation time was 5 min with stirring.

TFP was obtained from Boehringer Mannheim GmbH, DIDS from Fluka GmbH, Neu-Ulm, and LCC from Sigma Chemical Company.

Results

DIDS treatment

Fig. 1a show the time-course of absorption changes at 830 nm (ΔA_{830}) induced by repetitive laser flashes in oxygen-evolving PS II particles. The multiphasic relaxation kinetics of the signals are due to the P-680$^+$ re-reduction. An analysis of the data in Fig. 1a reveals that 67% of P-680$^+$ is reduced in the ns time-scale with half-life times of about 30 ns and 260 ns (see Table I). The elimination of oxygen evolution by incubation with 2 mM NH_2OH leads to an almost complete disappearance of the ns kinetics (Fig. 1b, Table I). In contrast to that a markedly different pattern arises if an almost complete inhibition of O_2-evolution capacity is achieved upon incubation of PS II particles with 2 mM DIDS (Table I). In this case, however, a similar retardation of the P-680$^+$ reduction is not observed (compare Fig. 1b and c). The dependence of the oxygen-evolution capacity and of the amplitude of the nanosecond component of P-680$^+$-reduction, $\Delta A^{ns}_{830} = \Delta A_{830}(t = 20$ ns$) - \Delta A_{830}(t = 900$ ns), upon the concentration of NH_2OH and DIDS is depicted in Fig. 2a and 2b, respectively. Within the scattering of the data a 1 : 1 correlation between O_2-evolution capacity and ΔA^{ns}_{830} is observed upon NH_2OH treatment (Fig. 2a). On the contrary Fig. 2b shows that the DIDS inhibitory response curves are clearly different for O_2 evolution and ΔA^{ns}_{830}. In order to assure that this remarkable difference is not due to any unconsidered deviations in the DIDS treatment, a number of experiments were performed under a different protocol. In this case at the beginning the average oxygen yield of a DIDS-treated sample was measured. Subsequently, this sample was transferred by a syringe to the cuvette of the flash photometer and the flash-induced 830 nm absorption changes were monitored. This protocol implies that the total DIDS incubation time for the ΔA_{830} measurements was about 5 min longer than for the O_2 measurements. Nevertheless, the difference between O_2-evolution capacity and ΔA^{ns}_{830} was nearly the same as in Fig. 2b.

Regardless of the detailed mechanism the data of Fig. 1a–c and Fig. 2 suggest a more specific site of DIDS interaction with the water-oxidizing enzyme system Y. This interaction eliminates the oxygen-evolution capacity without affecting the electronic and vibronic coupling between P-680$^+$ and Z. In this case a subsequent addition of NH_2OH to DIDS-treated samples is expected to cause a more severe modification which leads to the marked retardation of P-680$^+$ reduction. This was found to be the case. Single-flash experiments with a lower time resolution revealed that the full amplitude of ΔA_{830} with μs-relaxation kinetics are observed after the first flash in dark-adapted DIDS-treated particles in the presence of NH_2OH. In the subsequent flashes the amplitude of ΔA_{830} decreases while the kinetics remain unaffected (data not shown). This light-induced synergistic effect of DIDS and NH_2OH on the extent of the PS II turnover is probably related to limitations at the acceptor side and will not be considered here.

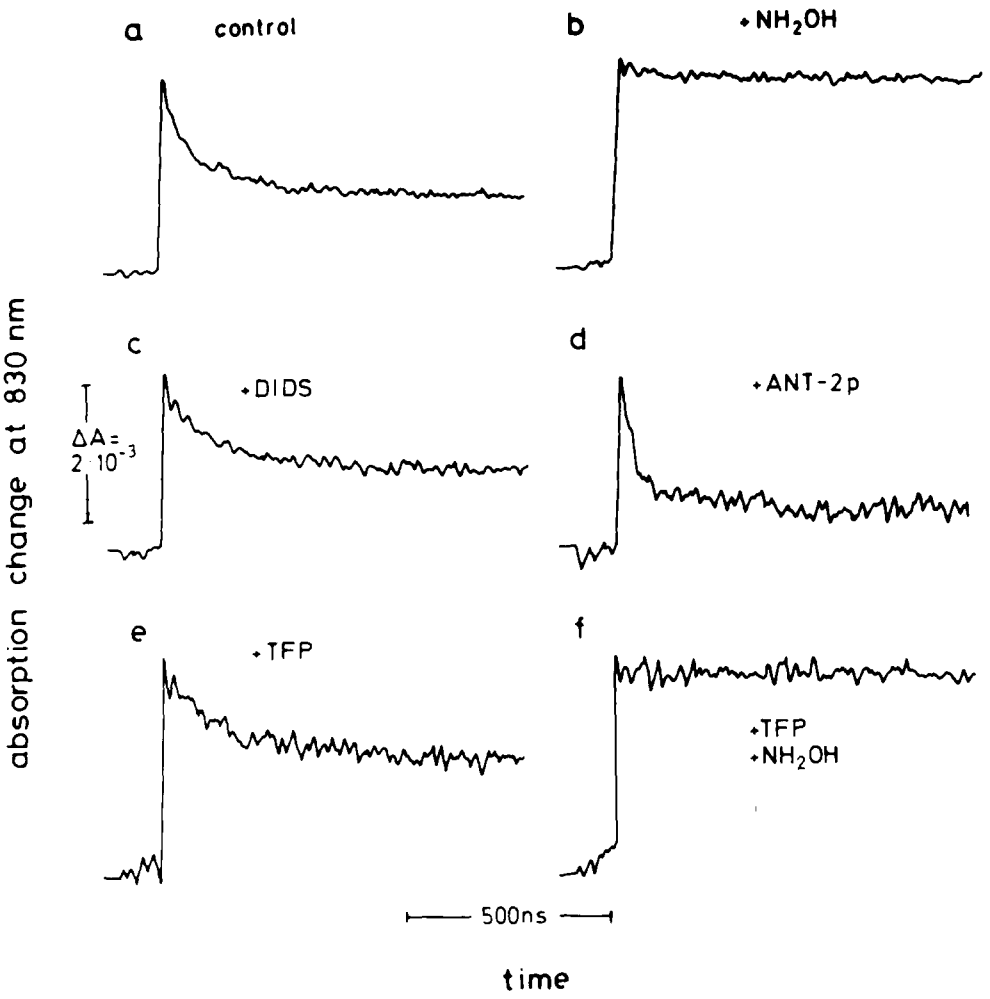

Fig. 1. Time-course of the absorption changes at 830 nm induced by repetitive laser flashes (repetition rate, 1.7 Hz) in PS II preparations in the presence of 1 mM $K_3Fe(CN)_6$ (control). Further additions as indicated in the figure: 2 mM NH_2OH, 2 mM DIDS, 500 nM ANT 2p, 0.2 mM TFP. The incubation time was 5 min for all compounds.

At the first glance a rather simple explanation could be offered for the appearance of ns kinetics in P-680$^+$ reduction even in the absence of observable oxygen evolution in DIDS-treated samples if one assumes that DIDS induces an ADRY-type effect [20]. ADRY agents are known to accelerate the deactivation of the S_2 and S_3 state of the water-oxidizing enzyme system Y [22] to the state S_1 [23]. Accordingly, under repetitive flash excitation at sufficiently low frequency the redox transitions of the water-oxidizing enzyme still occur but system Y lacks accumulation of oxidizing redox equivalents sufficient for O_2 formation. In order to test the possibility of a DIDS-induced ADRY-type effect we performed properly selected check-experiments. One possibility is offered by the dependence of the P-680$^+$ reduction kinetics on the redox state S_i of system Y with half-life times of about 25 ns in the state S_0 and S_1 and 50 ns and 250 ns in the S_2 and S_3 state [24]. Therefore, under repetitive flash excitation, an ADRY effect should lead to an acceleration of the P-680$^+$ reduction to a 25 ns kinetics due to the increased population of S_1. This is clearly observed after the addition of the most powerful and specific ADRY agent 2-(3-chloro-4-trifluoro-

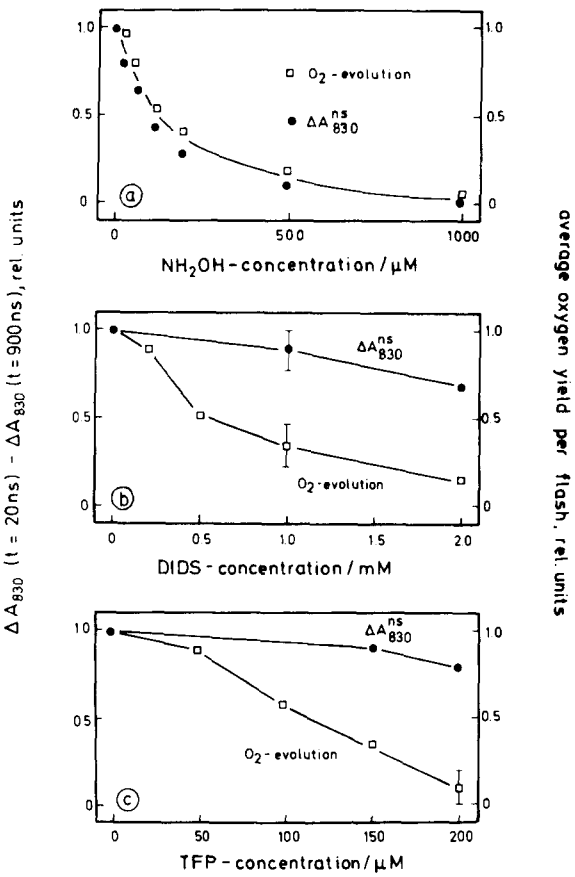

Fig. 2. Average oxygen yield er flash and the initial amplitude of the ns decay component of the absorption changes at 830 nm, ΔA^{ns}_{830}, as a function of the concentration of NH_2OH (a), DIDS (b) and TFP (c). Other conditions as in Fig. 1.

methyl)anilino-3,5-dinitrothiophene (ANT 2p) to the PS II particles (Fig. 1d). The addition of DIDS, however, leads to a slightly slower P-680$^+$ reduction kinetics (Fig. 1c). A more detailed analysis of the signals of Fig. 1 (see Table I) reveals that in the presence of ANT 2p the reduction kinetics are dominated by a component with a half-life time of about 20 ns. Furthermore, compared with the control the relative contribution of ns-kinetics to the overall relaxation of the 830 nm absorption changes is significantly larger in the presence of ANT 2p. This very interesting effect will be analyzed in a forthcoming paper.

In DIDS-treated samples the 260 ns component is hardly affected in comparison with the control and the 30 ns component is slightly retarded to about 60 ns. Dueto the limited signal-to-noise ratio this shift in the half-life time of the relaxation kinetics might be within the scattering of the data. Regardless of this kinetical detail a considerable decrease of the amplitude of the 30–50 ns component of ΔA_{830} is observed in DIDS-treated samples. The analysis of the relaxation kinetics summarized in Table I clearly shows that in contrast to typical ADRY agents DIDS treatment does not simply increase the reduction rate of S_2 and S_3 down to the redox state S_1 (and/or S_0). However, on the basis of these data a selective destabilization of the S_3 state down to S_2 cannot be excluded. In this case, a decrease of the dark time t_d between the actinic flashes should lead to an increase of oxygen yield. In contrast to the

TABLE I

HALF-LIFE TIMES AND RELATIVE AMPLITUDES OF THE P-680$^+$ REDUCTION KINETICS AND RELATIVE OXYGEN YIELD PER FLASH

The half-life times T_1, T_2 and the corresponding relative amplitudes A_1 and A_2 are the result of a two-exponential fit of the signals shown in Fig. 1. The slower components with decay times in the microsecond time-scale (T_3) were taken into account by the relative amplitude A_3. The amplitudes A_i are normalized to the initial amplitude of the control signal (trace a). Therefore, the sum of A_i is not 1.00 for samples treated with DIDS, TFP or ANT 2p. Average oxygen yield per flash was measured as described in Materials and Methods.

	Half-life times (ns)			Relative amplitudes (relative units)			Average oxygen yield per flash (%)
	T_1	T_2	T_3	A_1	A_2	A_3	
Control	30	260	>1000	0.43	0.24	0.33	100
+2 mM DIDS	57	270	>1000	0.25	0.20	0.41	7
+0.2 mM TFP	52	240	>1000	0.24	0.30	0.50	12
+500 nM ANT-2p	18	250	>1000	0.79	0.14	0.16	10
+2 mM NH_2OH	–	–	>1000	–	–	1.0	< 5

findings in the presence of ANT 2p (Fig. 3), however, in samples treated with 2 mM DIDS the oxygen yield is almost independent of t_d. These data are not easy to reconcile with an ADRY-type mechanism. In the control sample and at lower concentrations of DIDS and TFP a considerable drop of oxygen yield is observed, when t_d is decreased from 200 ms to 100 ms (Fig. 3). This is probably due to rate limitations on the acceptor side of PS II (see Ref. 25), leading to a limited extent of Q_A^- reoxidation between the actinic flashes at higher repetition rates. Therefore the data of Fig. 3 do not rule out a very fast decay of the S_3 state that is accomplished within a few milliseconds.

In order to test a possible effect of DIDS on the reoxidation of S_2 by Q_A^- measurements of the variable fluorescence were performed in DCMU-inhibited PS II samples as a function of the dark time after illumination with continuous light. The recovery of the variable fluorescence reflects the reoxidation of Q_A^- between the 1st and 2nd illumination. In correspondence with previous findings [26] ANT 2 p causes a marked suppression of the variable fluorescence in the second illumination (Fig. 4). On the other hand, the reoxidation of Q_A^- by S_2/S_3 seems not to be much affected by 2 mM DIDS, indicating that the S_2 and/or S_3 state is still available for recombination with Q_A^-. Therefore, the data of Fig. 3 and Fig. 4 supply sufficient evidence that at least the life-time of the S_2 state should not be markedly shortened by DIDS treatment.

Trifluoperazine treatment

Similar experiments as with DIDS were performed with the calcium antagonist TFP. Fig. 1e and Table I show that the TFP effects largely resemble those of DIDS. As in DIDS-treated samples mainly the 30–50 ns component of ΔA_{830} becomes modified while the 260 ns component remains nearly unchanged. Furthermore, the inhibitory response curves are clearly different for O_2 evolution and ΔA_{830}^{ns} (Fig. 2c). The only noticeable difference is the invariance of the initial amplitude of ΔA_{830} after addition of NH_2OH to TFP-treated samples even under repetitive flash excitation (Fig. 1f). However, this difference is not relevant for the topics to be discussed here and will not be further analyzed. As in the control samples, NH_2OH converts the nanosecond kinetics into microsecond kinetics without affecting the total amplitude of ΔA_{830} significantly.

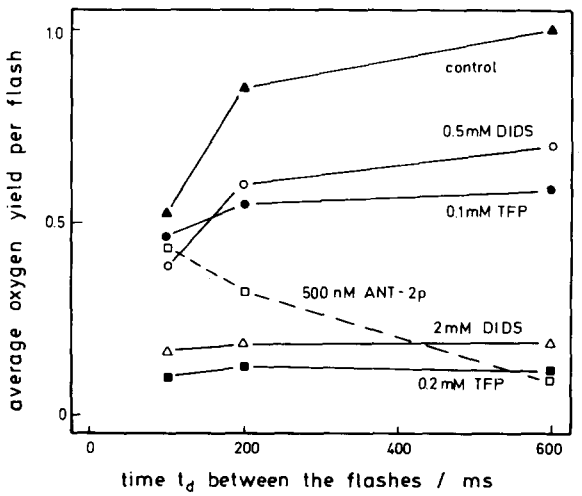

Fig. 3. Average oxygen yield per flash under repetitive flash excitation as a function of the dark time, t_d, between the flashes in PS II preparations in the presence of 1 mM $K_3Fe(CN)_6$. Further additions as indicated in the figure.

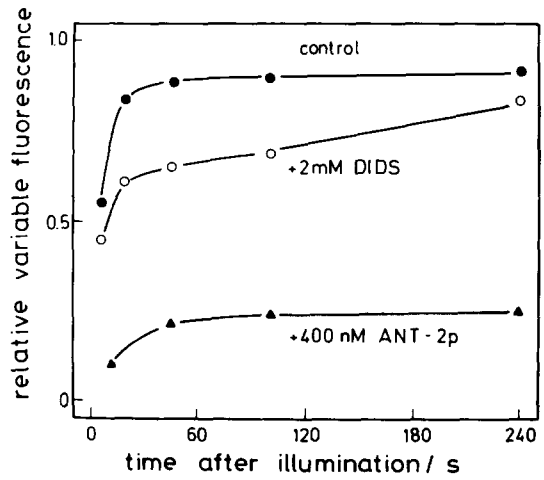

Fig. 4. The effect of ANT 2p and DIDS on the recovery of variable fluorescence after preillumination. Dark-adapted PS II samples were illuminated in the presence of 10 μM DCMU (control). After the indicated dark time the fluorescence induction was recorded. Further additions as indicated in the figure.

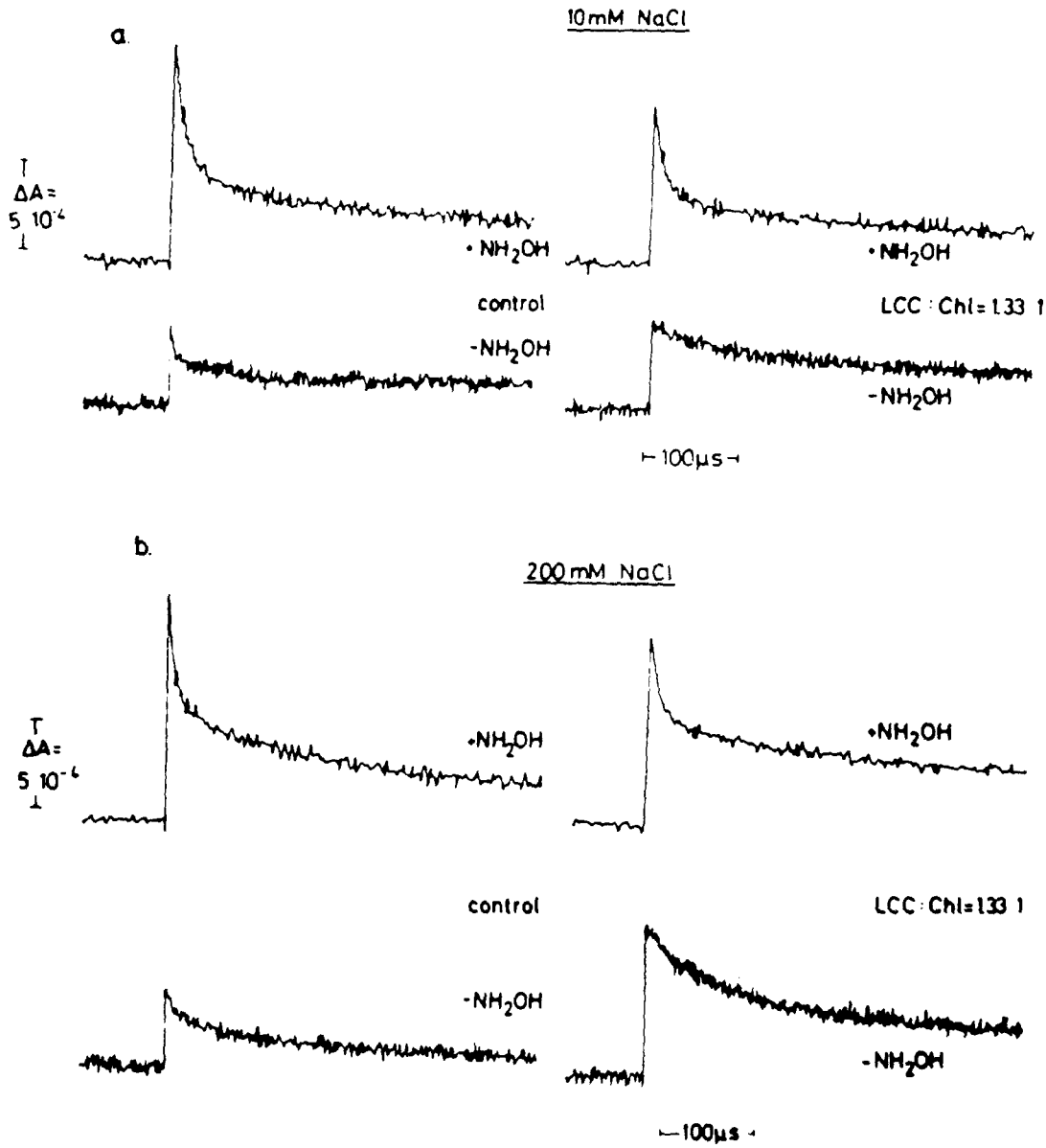

Fig. 5. Time-course of the absorption changes at 830 nm induced by repetitive laser flashes (repetion rate, 1.7 Hz) in untreated (control) and LCC-treated (LCC/Chl = 1.33 : 1) digitonin/CHAPS preparations measured with microsecond time resolution (see text for further details). The buffer media contained either 10 mM NaCl (a) or 200 mM NaCl (b). The upper traces in each panel were obtained after incubation of the sample with 3 mM NH_2OH for 6 min. Electron acceptor, $K_3Fe(CN)_6$.

Lauroylcholinechloride treatment

LCC treatment is another procedure to suppress oxygen evolution [15]. Unfortunately, due to enhanced scattering properties of the BBY preparations * treated with LCC, we were unable to measure the absorption changes at 830 nm with the high time resolution necessary to resolve the nanosecond kinetics. The scattering properties improved considerably by using the digitonin/CHAPS preparation (see Materials and Methods); however, even with these samples measurements with nanosecond time resolution were only poss-

* BBY preparations are called after the authors of Ref. 17.

ible at lower LCC concentrations with a somewhat poor signal-to-noise ratio. Therefore, to obtain ΔA_{830} measurements at higher LCC amounts and a complete concentration curve, we increased the signal-to-noise ratio by using a flash spectrophotometer in which the time resolution was limited to about 1 μs (see Materials and Methods). The nanosecond kinetics become undetectable at this time resolution and, therefore, the total initial amplitude (ΔA^{total}) cannot be determined. In order to circumvent this problem we used an indirect procedure for determining the relative extent of the ns kinetics as described recently [9,27]. Samples measured in the presence of 3 mM NH_2OH were used as reference. If one assumes that NH_2OH does not affect the extent of P-680$^+$ formation, the contribution of the nanosecond kinetics can be estimated from the difference of the signal amplitudes at 2 μs observed in samples with and without NH_2OH:

$$\Delta A^{\text{ns}*}_{830} = \Delta A^{2\mu s}_{830}(+NH_2OH) - \Delta A^{2\mu s}_{830}(-NH_2OH)$$

Fig. 5 shows typical traces of ΔA_{830} over a 500-μs time sweep for a digitonin/CHAPS preparation with and without LCC/Chl = 1.33 : 1 and 3 mM NH_2OH, in the presence of either 10 mM or 200 mM NaCl. The two different salt conditions were employed, since we have observed a different release of the 16, 23 and 33 kDa extrinsic polypeptides from PS II after LCC treatment as a function of NaCl concentration (data in preparation).

Upon addition of LCC one observes a decrease of $\Delta A^{\text{ns}*}_{830}$, while the total amplitude of ΔA_{830} in the presence of NH_2OH is only slightly affected. However, the decrease of $\Delta A^{\text{ns}*}_{830}$ is much less distinctive under low salt condition than under high salt condition. From Fig. 5 one calculates $\Delta A^{\text{ns}*}_{830}$ to be about 50% under low salt condition with respect to the appropriate control. On the other hand, the LCC-induced inhibition of O_2 evolution is practically independent of the salt concentration. At the identical LCC concentration and experimental conditions, the flash-induced O_2 evolution was reduced to about 25% under both the low and high salt condition with respect to the appropriate control. Fig. 6 shows a plot of $\Delta A^{2\mu s}_{830}(+NH_2OH)$, $\Delta A^{\text{ns}*}_{830}$, and the O_2 yield normalized to the control values under each condition as a function of LCC amount in the presence of either 10 mM NaCl (Fig. 6a) or 200 mM NaCl (Fig. 6b). For all of these measurements the same sample preparation was used under identical assay conditions including assay medium, Chl concentration, incubation time and mixing. At 10 mM NaCl the LCC inhibitory response curves are different for O_2 evolution, $\Delta A^{\text{ns}*}_{830}$, and $\Delta A^{2\mu s}_{830}(+NH_2OH)$ with the LCC amounts which induce a 50% loss in activity being roughly LCC/Chl = 0.5 : 1, 1.2 : 1 and 2 : 1, respectively. Interestingly, there is a loss in the total signal amplitude as well as in the amplitude of the nanosecond decay component of ΔA_{830}.

At 200 mM NaCl the difference in the inhibitory response for O_2 evolution and $\Delta A^{\text{ns}*}_{830}$ collapses, while the inhibitory response of $\Delta A^{2\mu s}_{830}(+NH_2OH)$ is little changed. The LCC amounts which induce a 50% loss in activity in

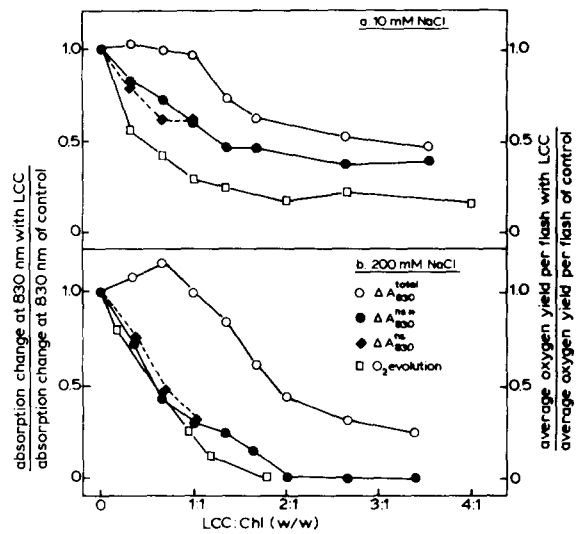

Fig. 6. Average yield per flash of the O_2 evolution (open squares), ΔA_{830} nanosecond decay components (i.e., $\Delta A^{\text{ns}*}_{830}$, closed circles) and ΔA_{830} total signal amplitude (i.e., $\Delta A^{2\mu s}_{830}(+NH_2OH)$, open circles) in a digitonin/CHAPS preparation as a function of the amount of LCC added in buffer media containing either (a) 10 mM NaCl or (b) 200 mM NaCl. $\Delta A^{\text{ns}*}_{830}$ was determined from measurements with μs time resolution as described in the text. The closed diamond symbols are data from direct measurements with nanosecond time resolution (i.e., $\Delta A^{\text{ns}}_{830}$, see text). Flash repetition rate, 1.7 Hz.

this case are roughly LCC/Chl = 0.5 : 1 for O_2 evolution and ΔA^{ns*}_{830}. For the sake of comparison the data points from the direct measurements of ΔA^{ns}_{830} up to LCC/Chl = 1 : 1 are also included in Fig. 6. These data closely parallel the behavior of ΔA^{ns*}_{830} in this LCC concentration range. This supports the assumption that ΔA^{ns*}_{830} can be used to determine the extent of the nanosecond components.

The above results clearly indicate that the various charge-transfer steps in PS II are differently affected by LCC depending upon the LCC concentration and salt conditions. However, the important point is, that similar to DIDS and TFP treatment, at low LCC amount and low salt condition O_2 evolution is considerably more suppressed then the extent of ΔA^{ns}_{830}.

Discussion

The results presented in this study clearly show that the initial amplitude of the nanosecond component of ΔA_{830}, ΔA^{ns}_{830}, is much less affected by treatments of PS II samples with DIDS, TFP or LCC than is flash-induced O_2 evolution. This indicates that under appropriate conditions these treatments lead to a state of PS II in which a significant fraction of P-680$^+$ is still reduced in the nanosecond time-scale without showing observable contributions to O_2 evolution. Hence, in contrast to NH_2OH- or Tris-treatment in which a 1 : 1 correlation between O_2 evolution and ΔA^{ns}_{830} is observed, under our conditions, inhibition of O_2 evolution by addition of DIDS, TFP or LCC does not affect the kinetics of the electron transfer from Z to P-680$^+$ in a large part of the reaction centers. Therefore, one has to assume that the site of inhibition of O_2 evolution by these compounds is closer to the O_2 evolving site. In agreement with this, subsequent addition of NH_2OH to samples treated with DIDS, TFP or LCC still leads to the retardation of the P-680$^+$ reduction kinetics to the microsecond time-scale.

The observed non-correlation between oxygen evolution capacity and relative extent of P-680$^+$ reduction in the ns range by treatments with DIDS, TFP or LCC cannot be simply explained by a normal ADRY-type effect with a destabilization of the S_2 and S_3 state of Y (see above). For TFP and DIDS one could consider an inhibition mode as in Cl^--depleted samples [28–30] in which the S-state advancement beyond S_2 is blocked leading to a slow biphasic re-reduction of Z^+ after the third actinic flash. In this case, even at a flash repetition rate of 1.7 Hz as used in the experiments of Fig. 1, according to the data of ref. 28 and 29, Z^+ should stay oxidized between the actinic flashes in about 50% of the reaction centers and the P-680$^+$ reduction kinetics should be slowed down to the microsecond time-scale in these reaction centers. However, this behaviour is not observed in samples treated with DIDS or TFP (Fig. 1). Furthermore, a preliminary set of experiments in DIDS-treated samples, which will be outlined in a forthcoming publication, indicates that an increase of the repetition rate (8 Hz) does not lead to a conversion of the ns-reduction kinetics of P-680$^+$ to the μs time-scale. Therefore, the specific blockage of a redox step in the water-oxidizing enzyme system Y ($Z^{ox}S_3 + 2H_2O \rightarrow ZS_0 + O_2 + 2H^+$) seems not to be probable. The persistence of the periodicity four oscillation of delayed fluorescence in LCC-treated thylakoids [15] suggests a repetitive turnover of the S_i-state-transitions without formation of dioxygen. An analogous 'uncoupling' of the dioxygen forming step could be caused by DIDS and TFP. Therefore the question arises about the mechanism of this uncoupling. If one assumes that S_3 becomes highly destabilized (i.e., a modified ADRY effect) then either an endogenous component or the exogenous substances (DIDS, TFP, LCC) could act as electron donor for a rapid reduction of S_3. The possibility of DIDS being a PS II donor can be excluded on the basis of preliminary experiments with samples which were washed three times after the DIDS treatment. According to spectroscopic measurements these preparations contained less than 5 DIDS molecules per PS II reaction center. But even after preillumination with 128 saturating flashes, the difference between O_2 evolution capacity and ΔA^{ns}_{830} was still observed (data not shown). Similar observations were established for washed LCC-treated samples. Furthermore, Carpentier and Nakatani [13] reported an inhibition of steady-state DCIP photoreduction only at much higher concentrations of TFP/Chl and DIDS/Chl, which, nevertheless, could be overcome by the PS

II electron donor diphenylcarbazide. And LCC itself does not act as a PS II donor after the Tris block [16]. Therefore, it appears very likely that one or more internal components act as electron source for a rapid S_3 decay even under repetitive excitation conditions. This implies questions about the mechanism of this proposed S_3 decay and the nature of endogenous donor(s). An interesting possibility arises if one accepts that in S_3 a peroxide-like structure does exist as proposed previously [31,32]. In this case the destabilization of this peroxide structure accompanied by the induction of a peroxidase activity could open an internal pool of substances to act as donors for a rapid S_3 decay. Experiments are in progress to analyze this hypothesis.

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