ISSN 0006-2979, Biochemistry (Moscow), 2007, Vol. 72, No. 11, pp. 1205-1215. © Pleiades Publishing, Ltd., 2007.
Published in Russian in Biokhimiya, 2007, Vol. 72, No. 11, pp. 1482-1494.
Originally published in Biochemistry (Moscow) On-Line Papers in Press, as Manuscript BM07-117, August 31, 2007.

Effect of Calcium Chelators on the Formation and Oxidation of the Slowly Relaxing Reduced Plastoquinone Pool in Calcium-Depleted PSII Membranes. Investigation of the F_0 Yield

B. K. Semin[1, 2]*, L. N. Davletshina[1], A. A. Bulychev[1], I. I. Ivanov[1], M. Seibert[2], and A. B. Rubin[1]

[1]*Department of Biophysics, Faculty of Biology, Lomonosov Moscow State University, 119991 Moscow, Russia; fax: (495) 939-1115; E-mail: semin@biophys.msu.ru*
[2]*Energy Sciences, National Renewable Energy Laboratory, Golden, Colorado 80401, USA; E-mail: mike_seibert@nrel.gov*

Received April 26, 2007
Revision received June 5, 2007

Abstract—The F_0 fluorescence yield in intact photosystem II (PSII), Ca-depleted PSII (PSII(−Ca/NaCl)), and Mn-depleted PSII membranes was measured before and after dim light treatment (1-2 min), using flash-probe fluorescence and fluorescence induction kinetic measurements. The value of F_0 after the light treatment (F_0') was larger than F_0 in dark-adapted PSII membranes and depended on the appearance of the slowly relaxing, reduced plastoquinone pool ($t_{1/2}$ = 4 min) formed during preillumination, which was not totally reoxidized before the F_0' measurement. In PSII(−Ca/NaCl) such a pool also appeared, but the F_0' yield was even higher than in intact PSII membranes. In Mn-depleted PSII membranes, the pool did not form. Interestingly, the yield of F_0' in Ca-depleted PSII membranes prepared using chelators (EGTA and citrate) or containing 5 mM EGTA was significantly lower than in PSII(−Ca/NaCl) samples prepared without chelators. These data indicate that chelators inhibit the reduction of Q_A and Q_B and formation of the slowly relaxing plastoquinone pool, or alternatively they increase the rate of its oxidation. Such an effect can be explained by coordination of the chelator molecule to the Mn cluster in PSII(−Ca/NaCl) membranes, rather than different amounts of residual Ca^{2+} in the membranes (with or without the chelator), since the remaining oxygen-evolving activity (~15%) in PSII(−Ca/NaCl) samples did not depend on the presence of the chelator. Thus, chelators of calcium cations not only have an effect on the EPR properties of the S2 state in PSII(−Ca/NaCl) samples, but can also influence the PSII properties determining the rate of plastoquinone pool reduction and/or oxidation. The effect of some toxic metal cations (Cd, Cu, Hg) on the formation of the slowly relaxing pool in PSII membranes was also studied.

DOI: 10.1134/S0006297907110065

Key words: photosystem II, oxygen-evolving complex, calcium, plastoquinone Q_A, plastoquinone Q_B, fluorescence, fluorescence induction kinetics, F_0

Photosystem II (PSII) is able to catalyze an important bioenergy reaction, water oxidation, accompanied by the evolution of molecular oxygen as a reaction by-product [1]. Synthesis of the intermolecular bond between the oxygen atoms of two oxidized water molecules during oxygen evolution is mediated by a catalytic center called the oxygen-evolving complex (OEC), incorporating four Mn cations and one Ca cation [2, 3]. One chloride anion is also involved in the water-oxidation reaction [4]. Perhaps the electron-transport component between the primary electron donor (P680) and the Mn cluster, tyrosine Y_Z residue (Tyr161 of the PSII reaction center D1

Abbreviations: F_0) minimal fluorescence of "open" reaction centers in dark-adapted samples; F_0') minimal fluorescence measured after preliminary exposure of a sample to light; F_{max}) maximum fluorescence yield; DPC) 1,5-diphenylcarbazide; FIK) fluorescence induction kinetics; OEC) oxygen-evolving complex; PSII) photosystem II; PSII(−Mn)) Mn-depleted photosystem II; PSII(−Ca/NaCl)) photosystem II with Ca extracted by treatment with 2 M NaCl; PSII(−Ca/NaCl + 50 μM EGTA)) photosystem II with Ca extracted by treatment with 2 M NaCl in the presence of 50 μM EGTA; PSII(−Ca/NaCl + 5 mM EGTA)) photosystem II with Ca extracted by treatment with 2 M NaCl in the presence of 5 mM EGTA; PSII(−Ca/NaCl + 5 mM EGTA → −EGTA)) photosystem II with Ca extracted by treatment with 2 M NaCl in the presence of 5 mM EGTA and with subsequent washing free of EGTA; PSII(−Ca/pH 3.0)) photosystem II with Ca extracted by treatment with citrate buffer (pH 3.0).

* To whom correspondence should be addressed.

protein), is also involved in water photolysis [5]. However, this hypothesis has not yet been proven.

Calcium is a necessary component of the OEC and is incorporated into the Mn_4/Ca cluster [2, 6]. According to X-ray diffraction analysis of the cyanobacterium *Thermosynechococcus elongates* PSII crystals with 3.0 Å resolution, Ca^{2+} is incorporated into a pyramidal structure with three Mn cations located 3.4 Å from the Ca^{2+} cation [7, 8] (measured by EXAFS [8, 9]). The catalytic center is at the inner surface of the PSII reaction center [7]. Access of soluble components in the medium to the catalytic center is limited by three peripheral proteins: PsbO (the 33-kD protein), PsbP (the 23-kD protein, in cyanobacteria PsbU (cytochrome *c*-550)), and PsbQ (the 17-kD protein, in cyanobacteria PsbV (12 kD)). The three proteins shield the Mn_4/Ca cluster [7]. The PsbP and PsbQ proteins mediate stabilization of the calcium cation (as well as the chloride anion) in the OEC [10]. Extraction of these proteins from the OEC (in the light, using 1-2 M NaCl solution) is accompanied by the extraction of one calcium cation [11]. Calcium extraction is due to a decrease in efficiency of cation binding [12].

Extraction of calcium from the OEC results in a significant modification of both the donor and acceptor side characteristics of PSII. It was shown that exposure of Ca-depleted PSII preparations to light, induced transfer of only two electrons from the donor side to the acceptor. In other words, the S-state cycle of the OEC is inhibited at the stage of the formal S3′ state in the configuration $S2Y_Z^{\bullet}$ [13-16]. The Ca-depleted PSII preparations are unable to evolve oxygen. The blockage of the S-state cycle is thought to be due to electron transfer inhibition at one of two donor sites on the donor side of the OEC electron-transport chain (OEC $\rightarrow Y_Z \rightarrow$ P680). It was shown that electron transfer from Y_Z to $P680^+$ was not inhibited [17-20]. In other words, the inhibition occurs at the site of Mn cluster oxidation by the $Y_Z^{\bullet}$ radical. This means that inhibition should result in a significant decrease in the rate of reduction of $Y_Z^{\bullet}$ (or complete inhibition of this process) by electrons from the Mn cluster. However, results of the measurement of $Y_Z^{\bullet}$ reduction in Ca-depleted PSII preparations are controversial, and are dependent on the method of Ca^{2+} extraction from the preparations (Ca^{2+} extraction without a chelator or use of a chelator for extraction and/or experiments). A significant decrease in the rate of reduction of the $Y_Z^{\bullet}$ radical (appearance of a slow kinetic species of the tyrosine radical EPR signal SII_{fast} [18, 19] and deceleration of decay of this radical after a light flash [17, 21]) were observed in experiments with chelators. In the absence of a chelator, the EPR signal SII_{fast} was not observed in Ca-depleted PSII preparations [17], because the time for $Y_Z^{\bullet}$ radical reduction was shorter than the time resolution of the EPR spectrometer (typical of intact PSII preparations). Similar results were obtained by Boussac et al. with EPR measurements in the absence of a chelator [20]. It should be noted that high concentrations of chelators (sodium citrate [22], EGTA [23] or EDTA [24]) are able to modify the EPR signal of the OEC S2 state by increasing the lifetime of the state ($t_{1/2}$ = 7 h at 20°C [24]). It was shown in [25], that modification of the Mn cluster properties in this case was due to chelator-molecule binding to the Mn cluster. Perhaps chelator-molecule binding to the Mn cluster causes deceleration of electron transport from the Mn cluster to $Y_Z^{\bullet}$ radical.

In addition to significant changes on the donor side of PSII, calcium extraction is also accompanied by substantial modification of the acceptor side. For example, calcium extraction causes a significant increase (~150 mV) in the redox potential of the Q_A/Q_A^- couple [26]. This may have a significant effect on electron transport from Q_A^- to Q_B [27]. Perhaps this factor underlies the fact that the thermoluminescence band, corresponding to charge recombination within the $S2Q_A^-$ pair in Ca-depleted PSII membranes, is shifted toward higher temperature (45-55°C) [24, 27, 28]. However, some authors attributed this fact to recombination of Q_A^- with the $Y_D^{\bullet}$ radical rather than the S2 state of the Mn cluster [28]. Nevertheless, regardless of the nature of the electron acceptor involved in recombination, these results indicate that the lifetime of the reduced quinone (Q_A^-) in Ca-depleted PSII preparations is significantly longer than in intact PSII preparations. EPR studies revealed that the $t_{1/2}$ of Q_A^- oxidation in Ca-depleted PSII preparations was ~10 min [28]. It should be noted that the Ca-depleted PSII preparations used in these experiments were prepared using EGTA. In preparations obtained in the absence of a chelator, the properties on the acceptor side of PSII (fluorescence yield and decay kinetics) differed from those in preparations obtained in the presence of a chelator [17].

The data available from the literature show that chelators used for calcium extraction from PSII may modify not only the EPR properties of the Mn cluster [22-25], but also have an effect on the characteristics of electron transport on both donor [17, 20] and acceptor [17] sides of Ca-depleted PSII. The goal of our work was to study the stability of reduced plastoquinones in Ca-depleted PSII preparations (with the calcium extracted using different methods) using fluorescence detection methods. It is shown that Ca-depleted PSII membranes prepared without a chelator after preliminary exposure to dim light contain a slowly reoxidizing, reduced plastoquinone pool whose size is larger than the size of a similar pool observed under similar conditions in intact PSII preparations. It is also significantly larger than the pool in PSII(−Mn) preparations. The size of the pool significantly decreases if a chelator of calcium cations (EGTA or sodium citrate) is used for calcium extraction or if EGTA is added to Ca-depleted PSII membrane fragments. Thus, in addition to the effect on the EPR signal of S2 [22-24], chelators can significantly modify the reduction/oxidation of Q_B. We also suggest that the

method of detection of the slowly oxidizing plastoquinone pool could be used for elucidation of OEC-inactivation mechanisms under exposure to various factors and agents, including environmentally hazardous factors (e.g., metal cations).

MATERIALS AND METHODS

PSII membrane preparations (BBY-type) were isolated from market spinach as described in the literature [29, 30]. Isolated preparations were suspended in buffer A (0.4 M sucrose, 50 mM MES, 15 mM NaCl, pH 6.5) at chlorophyll (Chl) concentrations of 4-5 mg/ml and stored under liquid nitrogen. The oxygen-evolving activity of the BBY membranes, as measured polarographically using a Clark electrode, ranged from 400 to 500 μmol O_2/h per mg Chl when 0.2 mM 2,6-dichloro-*p*-benzoquinone was used as an artificial electron acceptor. Total concentration of Chl (*a* + *b*) in the preparations was measured using the methods of Porra et al. [31] or Arnon [32] in 80% acetone extracts.

Calcium from the PSII OEC was extracted using a high-concentration NaCl solution in the absence [24] or in the presence of 50 μM EGTA [13, 24] or using citrate buffer (pH 3.0) [33]. According to the method described in [24], a buffer solution containing 2 M NaCl, 0.4 M sucrose, and 25 mM MES (pH 6.5) was used. The PSII preparations were incubated in this solution at 0.5 mg/ml Chl concentration for 15 min under dim light at room temperature. The resulting preparation was washed twice with buffer A to remove excess NaCl and resuspended in buffer A (PSII(−Ca/NaCl)). According to the method described in [13], incubation of PSII preparations in 2 M NaCl/50 μM EGTA solution for 15 min was followed by washing and resuspending in buffer A containing 50 μM EGTA [PSII(−Ca/NaCl + 50 μM EGTA)]. In addition, preparations obtained by Ca^{2+} extraction with NaCl followed by the addition of 5 mM EGTA [24] were used in some experiments. Furthermore, some of these preparations [PSII(−Ca/NaCl + 5 mM EGTA)] were washed and resuspended in buffer A containing no EGTA [PSII(−Ca/NaCl + 5 mM EGTA → −EGTA)]. According to the method of Ono and Inoue [33], PSII preparations were washed free from MES buffer using medium containing 0.4 M sucrose and 20 mM NaCl. The resulting preparation was suspended in citrate buffer (0.4 M sucrose, 20 mM NaCl, 10 mM sodium citrate, pH 3.0) at a Chl concentration of 2 mg/ml, incubated for 5 min in the dark on ice, and diluted with buffer A to pH 6.5 [PSII(−Ca/pH 3.0)].

Manganese from the PSII OEC was extracted by incubation of PSII membranes (0.5 mg Chl per ml) in 1.0 M Tris-HCl buffer (pH 9.4) containing 0.4 M sucrose for 30 min under dim light at 5°C. The resulting preparation was washed twice with buffer A [34]. The residual oxygen-evolving activity after treatment with Tris was 5% of the initial level.

Fluorescence yield kinetics after a saturating light flash were measured at room temperature using the laboratory set-up described by Ghirardi et al. [35]. A xenon flash lamp was used as the source of saturating light flashes (pulse duration, 3 μsec) to induce charge separation in the PSII reaction centers. Probe light flashes of low intensity were generated using Hewlett-Packard (USA) light-emitting diodes (LEDs). Fluorescence yield kinetics were measured as follows: the yield of fluorescence induced by the probe light flashes was measured for 2 sec prior to application of the saturating light flash (fluorescence yield F_0 for open PSII reaction centers). Then, the saturating light flash was applied, and the content of closed reaction centers (probe flashes induce the fluorescence) was measured for 3 sec. Initially, the fluorescence yield curve is represented as a fluorescence intensity in volts. After that, the results of the measurements were normalized to F_0 and represented on a relative scale $(F - F_0)/F_0$.

Fluorescence induction kinetics (FIK) were measured using a portable Plant Efficiency Analyser (Hansatech Instruments Ltd., UK). LED sources of excitation light (λ_{max} = 650 nm; spectral range, 580-710 nm) were used in the fluorimeter. The time resolution of fluorescence detection was 10 μsec (within the initial 2 msec); 1 msec (within the time interval from 2 msec to 1 sec); and 100 msec (time interval >1 sec). Fluorescence induction kinetics were measured at saturating actinic (1200 μE/m^2·sec) intensity of the excitation light flux. The fluorescence signal at 50 μsec after application of continuous actinic light was defined as F_0, because the fluorescence yield at that time did not depend on the exciting light intensity [36]. Therefore, the initial moment of fluorescence detection in the figures corresponds to 50 μsec. A logarithmic time scale was used in the figures as it is commonly employed for the presentation of fluorescence induction kinetics.

RESULTS AND DISCUSSION

Removal of the extrinsic proteins using a high-concentration NaCl solution (1-2 M) results in 70-90% inhibition of the oxygen-evolution reaction [10, 13, 37]. The activity of the treated preparations can be restored up to 80% of the initial level by adding high concentrations (10-20 mM) of calcium in the presence of chloride anions [10, 38]. It was shown that inhibition of PSII-preparation activity induced by washing with high-salt is actually due to the removal of calcium rather than the extrinsic proteins [12]. In other words, this method can be regarded as a method of calcium extraction from the OEC. A modification of this method employs the combination of a high-concentration NaCl solution and a chelator of calcium cations (EGTA or EDTA). Different ways of combining

the chelator and NaCl-washing procedures can be used during Ca-depletion. For example, 50 μM EGTA can be added to the medium after incubation of the preparation in NaCl solution and experiments with the samples are carried out in the presence of the same chelator concentration [13]. Sometimes higher chelator concentrations (5 mM) are added to the medium at the end of the NaCl incubation with an additional incubation for 5 min [24]. Measurements in the latter case were performed in the presence of 0.2 mM EDTA [24]. NaCl and EGTA can also be removed from the preparations by dialysis, giving rise to calcium-depleted PSII preparations containing the PsbP and PsbQ extrinsic proteins [23]. In our experiments, Ca-extracted PSII membrane preparations were prepared by different methods (with or without chelators). The oxygen-evolution activities of the different preparations are given in Table 1. It follows from the table that removal of Ca^{2+} is accompanied by a significant decrease in the rate of the water-oxidation reaction (residual activity, 10-20%), whereas the extent of inhibition is virtually independent of the addition of low (50 μM) or high (5 mM) concentrations of EGTA. These results are consistent with the literature [13, 24, 39]. The addition of calcium to PSII preparations with a calcium-depleted OEC caused a 70-80% restoration of the initial oxygen evolving activity (Table 1). These findings are also consistent with the literature [10, 38].

Table 1. Oxygen-evolving activity of different PSII preparations

PSII preparation	Oxygen-evolving activity, %
PSII	100*
PSII(−Ca/NaCl)	12
PSII(−Ca/NaCl) + 30 μM $CaCl_2$	75**
PSII(−Ca/NaCl + 50 μM EGTA)	13
PSII(−Ca/NaCl + 5 mM EGTA)	8
PSII(−Ca/NaCl + 5 mM EGTA) + + 30 mM $CaCl_2$	74
PSII(−Ca/NaCl + 5 mM EGTA → → −EGTA)	20
PSII(−Ca/pH 3.0)	11
PSII(−Ca/pH 3.0) + 50 mM $CaCl_2$	80
PSII(−Mn)	5

Note: The results given in the table represent the values averaged over 2-5 measurements. Standard deviations do not exceed 5% of the mean value.
* 100% corresponds to 400-500 μmol O_2/mg Chl per h.
** Samples (20 μg Chl per ml in buffer A at pH 6.5) were incubated in the presence of calcium for 15 min in the dark at 4°C.

Table 2. Fluorescence yield in different PSII preparations before (F_0) and after (F_0') preliminary illumination

Preparation	F_0, V*	F_0', V**
PSII	0.80	1.30 (1.6)***
PSII(−Ca/NaCl)	0.90	1.87 (2.1)****
F_0' without DCMU	0.90	1.85 (2.1)
0.5 mM FeCN and then DCMU were added after illumination	0.90	0.97 (1.1)
DCMU and then 0.5 mM FeCN were added after illumination	0.90	1.02 (1.1)
DCMU was added before illumination	0.90	1.41 (1.6)
PSII(−Mn)	0.75	0.85 (1.1)
illumination in the presence of 200 μM DPC	0.73	2.15 (2.9)

Note: The F_0 yield was measured using the flash-probe fluorescence method (see "Materials and Methods"). The results given in the table represent values averaged over 2-4 measurements. Standard deviations do not exceed 5% of the mean value.
* Concentrated membrane preparations, incubated for more than 1 h in the dark on ice, were suspended in buffer A to a concentration of 25 μg Chl per ml, and the fluorescence kinetics were measured in the presence of 40 μM DCMU (except the case in bold).
** Samples containing membrane preparations at a Chl concentration of 25 μg/ml were illuminated with dim light (~5 $\mu E/m^2 \cdot sec$) for 1 min, then incubated in the dark for 30 sec, 40 μM DCMU was added, and then subjected to a fluorescence yield measurement.
*** The factor of the F_0' yield increase relative to F_0 is given in parentheses.
**** As the period of dark incubation was increased to 4 min, $F_0' = 1.42$ or $F_0' = 1.15$ at incubation temperature 22 or 40°C, respectively.

Measurements of the fluorescence yield kinetics with PSII(−Ca/NaCl) preparations in the presence of 40 μM DCMU after a saturating light flash revealed: i) a substantial decrease in the F_{max} (Fig. 1) as compared to intact PSII preparations; ii) that the F_0 yield remains virtually unchanged upon the calcium extraction (Table 2); and iii) the fluorescence yield decay kinetics contained an initial stage of rapid fluorescence decrease (Fig. 1). After this stage was over, the rate of fluorescence yield (F) decreased significantly, and was substantially lower than that in intact PSII membrane fragments. The addition of EGTA to calcium-depleted preparations had virtually no effect on fluorescence kinetics. However, the results of our experiments differ slightly from the data obtained by

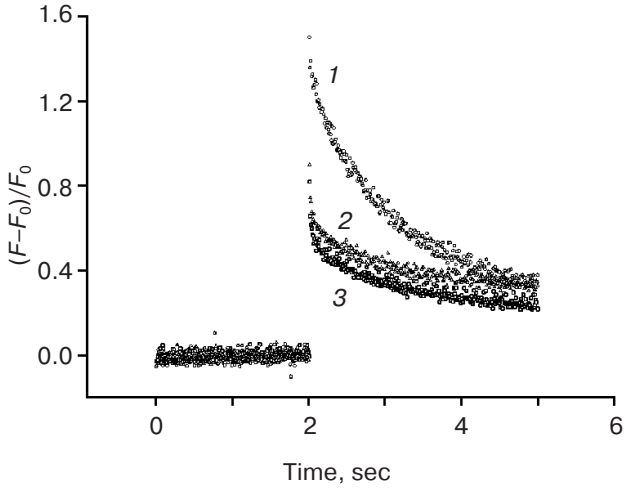

Fig. 1. Kinetics of fluorescence yield decay in intact PSII membrane preparations (*1*) and calcium-depleted PSII preparations (*2*, *3*). Concentrated preparations were incubated in the dark on ice for no less than 1 h, suspended in buffer A at a concentration of 25 μg Chl per ml before measurement, and then 40 μM DCMU was added. Calcium cations were extracted from the OEC by washing the PSII preparations with 2 M NaCl in the absence (*2*) or presence of 5 mM EGTA (*3*).

Andreasson et al. [17], who found that the fluorescence yield decay kinetics decelerated in the presence of 10 mM EGTA as compared to PSII preparations washed with NaCl containing no EGTA. Perhaps this discrepancy is due to the fact, that fluorescence in our experiments was excited using a single light flash, whereas Andreasson et al. reported fluorescence kinetics averaged over 10 light flashes. Additionally, in contrast to Andreasson et al., we measured fluorescence kinetics in the presence of DCMU. However, the fluorescence (F_{max}) amplitude decrease in Ca^{2+}-depleted PSII preparations could be interpreted similarly to Andreasson et al. [17], i.e., who attributed it to an increase in the rate of recombination between a fraction of the Q_A^- pool and $P680^+$. This suggestion is supported by the fact that the fluorescence yield decay kinetics in calcium-depleted PSII preparations contains an initial segment of rapid fluorescence decrease (Fig. 1).

Measurements of the fluorescence yield decay kinetics in PSII(−Ca/NaCl) preparations revealed that the size of the slowly oxidizing pool Q_A^- in the preparations was larger than in intact preparations. The kinetic characteristics of the pool were studied using the following approach. As the yield of the fluorescence F (rather than F_0) depends mainly on the concentration of Q_A^-, the Q_A^- concentration can be probed by measuring the intensity of the fluorescence induced by the weak light flashes used for measuring the F_0 yield. In additional experiments, the F_0 yield was measured in different PSII preparations, exposed for a short time (1-2 min) to low-intensity light (dim light, ~5 μE/m^2·sec) after a short-term (1 min) dark incubation. In fact, under these experimental conditions, the intensity of the fluorescence induced by the weak light flashes is not an actual F_0 level. Therefore, this value was designated as F_0'.

It follows from Table 2 that after preillumination of intact PSII preparations with weak light, the F_0' yield increased significantly (1.6 times that of F_0). Since the fluorescence intensity was measured a significant period of time after illumination (1 min) in preilluminated intact PSII preparations, either there is a slowly reoxidizing Q_A^- pool (slow oxidation may be caused by the inability of charge recombination to occur between Q_A^- and the OEC states, S0 and S1, and simultaneously the inability of its oxidation by plastoquinone Q_B, because Q_B has already been reduced as a result of the illumination), or there are other unidentified causes. In PSII(−Ca/NaCl) preparations (only NaCl was used to remove Ca^{2+}), the F_0' value was larger than in intact PSII preparations and 2.1 times larger than F_0. The addition of ferricyanide, an artificial electron acceptor, inhibits the increase in the F_0' value virtually completely (Table 2). The kinetics of the F_0' yield decrease during incubation of PSII(−Ca/NaCl) preparations in the dark after preillumination is shown in Fig. 2 (the fluorescence yield decrease $t_{1/2} = 4$ min at room temperature). An increase in the incubation temperature of the illuminated PSII(−Ca/NaCl) preparations to 40°C (a temperature close to the thermoluminescence peak temperature in PSII(−Ca/NaCl) preparations corresponding to recombination of Q_A^- with the S2 state of the OEC [24, 27, 28]) induces a significant increase in the rate of F_0' yield decay.

Preillumination of the PSII preparations with both calcium and manganese extracted (Table 2) has virtually

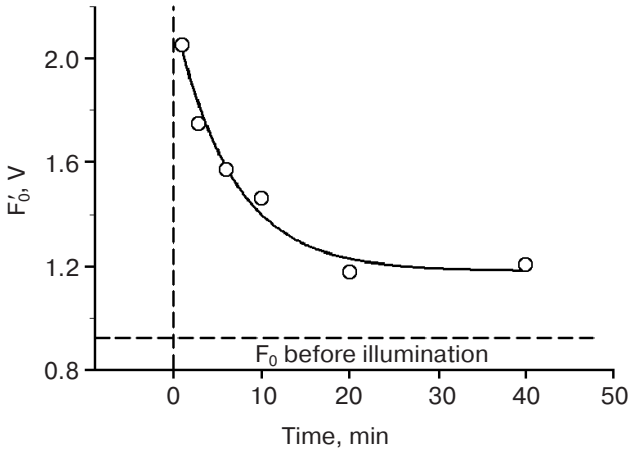

Fig. 2. Kinetics of the F_0' fluorescence yield decrease during dark incubation of preilluminated PSII(−Ca/NaCl) preparations at room temperature. PSII(−Ca/NaCl) membrane preparations, suspended in buffer A at 25 μg Chl/ml, were exposed to dim light (~5 μE/m^2·sec) for 1 min, incubated in the dark for the specified time interval, 40 μM DCMU was added, and the sample was subjected to an F_0' fluorescence yield measurement.

no effect on the F_0' yield in contrast to the native PSII or PSII(−Ca/NaCl) preparations. This can be explained in two ways: i) lack of an electron donor for $Y_Z^{\bullet}$ (formed as the result of charge separation) giving rise to one-electron reduction of the plastoquinone on the acceptor side of PSII, or ii) a high rate of recombination between $Y_Z^{\bullet}$ and Q_A^- (20-30 msec) [35, 40]. Indeed, if before preillumination of the PSII(−Mn) preparation, the artificial electron donor 1,5-diphenylcarbazide (DPC) capable of reducing $Y_Z^{\bullet}$ was added to the preparation, this would cause a significant increase (2.9 times, Table 2) in the F_0' fluorescence yield. This is mainly due to maximum reduction of the plastoquinone pool (because of the presence of the electron donor) and inhibition of recombination oxidation of Q_A^- (Y_Z is reduced). Note that DCMU was not present during preillumination.

The F_0' yield in various PSII preparations (including PSII preparations with the calcium extracted using different methods) after preillumination with weak light was also studied using another method of fluorescence detection. This method allows the F_0 yield to be measured by monitoring the fluorescence induction kinetics (FIK). This method of fluorescence yield measurement includes exposure of the preparation to saturating (this was tested in a preliminary experiment (not shown)) continuous light (1200 $\mu E/m^2 \cdot sec$). As in previous experiments, the fluorescence yield was measured before and after short-term dim illumination (2 min). The fluorescence was also measured in the absence and presence of DCMU added to the preparation after preillumination, but before FIK detection. The kinetics of fluorescence induction are shown in Fig. 3 for four types of PSII preparations, i.e., intact PSII, PSII(−Ca/NaCl) with Ca^{2+} extracted using a high-concentration NaCl solution, PSII(−Ca/NaCl) in the presence of 5 mM EGTA, and PSII without an OEC in the presence and absence of the exogenous electron donor DPC.

The PSII preparations not subjected to preillumination were characterized by the following specific features of the FIK. The shape of the FIK curve in intact PSII preparations contained three phases, O-J-P (the shoulder I in the curve is absent [41-43]) (Fig. 3a, curve *1*). The addition of DCMU, an inhibitor of primary plastoquinone Q_A^- oxidation by the secondary plastoquinone, Q_B, before the FIK measurement, is characterized by the disappearance of the J shoulder and by monotonic fluorescence saturation (Fig. 3b, curve *1*). The monotonic fluorescence saturation represents the reduction of the primary Q_A plastoquinone alone. The saturated fluorescence yield in this case is substantially lower than F_{max} in the absence of DCMU (compare curves *1* in Figs. 3a and 3b). DCMU had no effect on the F_0 yield. However, our experiments revealed that very weak light during DCMU addition could elevate F_0 (data not shown). The fluorescence decrease in the presence of DCMU (Fig. 3b, curve *1*) is characteristic of PSII preparations and can be attributed to the fact that fluorescence amplitude at the J shoulder corresponds to the maximum concentration of reduced Q_A [41, 43]. The J-P fluorescence yield increase phase is interpreted as reduction of the mobile plastoquinone pool (including complete Q_B reduction), since oxidized mobile plastoquinone pool is a fluorescence quencher [41, 43]. It should be noted, however, that the effects of DCMU on the FIK in leaves and PSII thylakoid membrane preparations are substantially different from each other [44].

In calcium-depleted PSII preparations obtained without a chelator [PSII(−Ca/NaCl)], the O-J-P transient is well pronounced (Fig. 3c). The FIK curve shape in this case is similar to the FIK curve in intact PSII preparations, which indicates effective OEC activity [41] and can be regarded as evidence for saturation of the entire plastoquinone pool on the acceptor side of PSII (Q_A and Q_B) with electrons. The effect of DCMU on the FIK in this case is similar to the DCMU effect in PSII preparations.

The addition of 5 mM EGTA to calcium-depleted PSII preparations (which has been used for measuring various characteristics of calcium-depleted PSII [17, 24]) gives rise to a substantial modification of the FIK curve shape (Fig. 3e). The FIK shape in this case differs significantly from the FIK shape in PSII(−Ca/NaCl) preparations (Fig. 3c). The FIK curve shape in [PSII(−Ca/NaCl) + EGTA] preparations (Fig. 3e) is evidence for insufficient electron flow to the acceptor side of PSII from the donor side of PSII (the J-P phase is absent and O-J phase is minimal) and chelator-induced inhibition of electron transfer from Q_A^- to Q_B (DCMU causes similar modifications of the FIK curve).

In manganese-depleted PSII preparations (electron flow to the acceptor side of PSII from the donor side of PSII is absent), the FIK curve contains a peak (Fig. 3g, curve *1*) typical of Mn-depleted PSII preparations, called peak K [36, 41]. Subsequent to K the fluorescence yield declines as the result of decreasing Q_A^- concentration. The Q_A^- concentration decrease is due to electron transfer from Q_A^- to Q_B and the lack of electron flow from the donor side of PSII (Fig. 3g) [45]. Thus, the FIK curve shape in PSII(−Ca/NaCl) preparations (Fig. 3c, *1*) more closely resembles intact PSII preparations than PSII preparations with an inhibited electron transport system [PSII(−Mn)]. Addition of a chelator to calcium-depleted PSII preparations [PSII(−Ca/NaCl)] has a significant effect on the FIK curve shape. These findings are consistent with the literature. Indeed, it was shown in [17-19, 21], that in PSII preparations with chelator-mediated calcium depletion electron transfer from the manganese cluster to Y_Z was inhibited. Thus, the FIK curve shapes in calcium depleted PSII preparations with or without EGTA substantially differ from each other. Chelators used for manganese extraction have a significant effect on not only the EPR properties of the S2 state [22-24], but

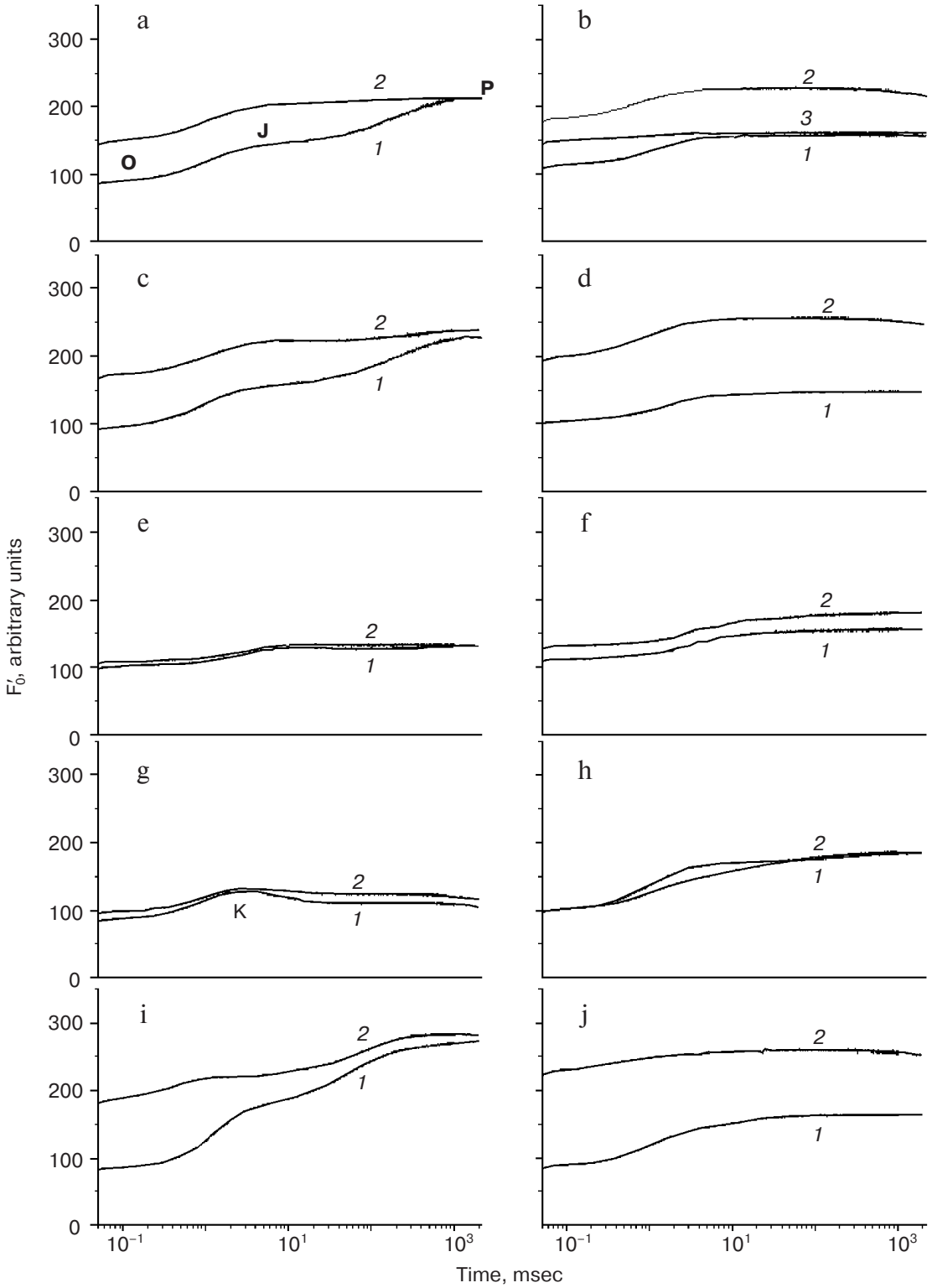

Fig. 3. Kinetics of fluorescence induction (FIK) in PSII preparations before (*1*) and after preillumination (*2*). a) PSII; c) PSII(–Ca/NaCl); e) PSII(–Ca/NaCl + 5 mM EGTA); g) PSII(–Mn); i) PSII(–Mn) + 200 μM DPC. Panels (b), (d), (f), (h), and (j) are similar to panels (a), (c), (e), (g), and (i) but DCMU was added to the samples after preillumination, but prior to the FIK measurement. Concentrated PSII membrane preparations were suspended in buffer A at 25 μg Chl per ml. Kinetics were measured in the presence of 40 μM DCMU before (*1*) and after (*2*) preillumination with dim light (~5 $\mu E/m^2 \cdot sec$) for 2 min (*2*). FIK (*3*) in panel (b) was measured in PSII preparations preilluminated after addition of 40 μM DCMU.

also on the PSII properties represented in the FIK curve shape.

Preillumination of PSII preparations with dim light also has a substantial effect on the FIK characteristics. When the FIK curve shape indicated the presence of electron transfer in PSII preparations, i.e. PSII (Fig. 3, a and b), PSII(−Ca/NaCl) (Fig. 3, c and d), and PSII(−Mn + DPC) (Fig. 3, i and j), the F_0' yield increased significantly (almost 2 times relative to F_0), and the magnification factors measured using the two different fluorescence methods (Tables 2 and 3) virtually coincided with one another. The FIK curve shapes before and after preillumination were also different. The fluorescence intensity in preilluminated preparations increases to the maximum P level within the time interval characteristic of the O-J transition. A similar effect was reported by Pospisil and Dau [41]. The absence of the J-P transition was attributed to the fact that preillumination induces reduction of the plastoquinone pool (the J-P transition in PSII preparations is characterized by the reduction of this pool) [41] (the oxidized pool is a fluorescence quencher). In other words, the plastoquinone pool was reduced before the FIK measurement. The F_{max} level of preilluminated PSII preparations, measured in the presence of DCMU (Fig. 3b, curve *2*), is substantially higher than in non-illuminated preparations (Fig. 3b, curve *1*). This result can be attributed to the fact that the plastoquinone pool in preilluminated preparations had already been reduced before the FIK measurement. It is impossible to further reduce this pool, because DCMU was added to non-illuminated preparations before the FIK measurement. On the other hand, the FIK curve shape measured with PSII preparations preilluminated in the presence of DCMU (Fig. 3b, curve *3*) indicates that 1-min dark incubation of illuminated PSII preparations brings Q_A back to the oxidized state, because the magnitude of the fluorescence intensity increase during O-P transition (Q_A reduction) is similar to that in non-illuminated preparations.

Thus, the results of this work can be regarded as evidence that the F_0 (F_0') yield increase after preillumination of the PSII preparations can be attributed to plastoquinone Q_B reduction (formation of a reduced plastoquinone pool). This results in the disappearance of the fluorescence quencher. The F_0' fluorescence yield decreases during dark incubation of the preilluminated preparations with a $t_{1/2} = 4$ min (Fig. 2). This time is close to the lifetime of reduced Q_B^- ($t_{1/2} = 3$ min [46]) and is also evidence attributing the F_0' yield increase to the presence of a reduced plastoquinone pool in preilluminated PSII preparations. The pool reduction is observed in both PSII preparations and PSII(−Ca/NaCl) preparations (see below), because preillumination of calcium-depleted PSII preparations (Fig. 3, c and d) modifies the FIK curve shape as measured in the absence (Fig. 3c) or pres-

Table 3. F_0 and F_{max} fluorescence yield levels in different PSII preparations before and after preillumination measured using the fluorescence induction kinetics detection method

Preparation	Fluorescence parameters, arbitrary units						
	before illumination*			after illumination**			
				F_0'			
	F_0	F_{max}	F_V/F_{max}	arbitrary units	magnification factor	F_{max}	F_V/F_{max}
PSII	110	159	0.308	178	1.62	228	0.219
PSII(−Ca/NaCl)	100	148	0.324	196	1.96	256	0.234
PSII(−Ca/NaCl + 50 μM EGTA)	107	144	0.256	136	1.27	165	0.175
PSII(−Ca/NaCl + 5 mM EGTA)	107	154	0.305	124	1.16	173	0.283
PSII(−Ca/NaCl + 5 mM EGTA → −EGTA)	99	142	0.303	146	1.47	182	0.199
PSII(−Ca/pH 3.0)	97	136	0.288	137	1.41	159	0.138
PSII(−Mn)	97	183	0.472	98	1.01	186	0.471
PSII(−Mn) + 200 μM DPC	83	201	0.587	225	2.71	260	0.135

Note: The results given in the table represent values averaged over 2-5 measurements. Standard deviations do not exceed 8% of the mean value.

* Concentrated PSII membrane preparations incubated for more than 1 h in the dark on ice were suspended in buffer A at a concentration of 25 μg Chl per ml, and the fluorescence kinetics were measured in the presence of 40 μM DCMU.

** Samples containing membrane preparations with a Chl concentration of 25 μg/ml were illuminated with dim light (~5 $\mu E/m^2 \cdot sec$) for 2 min, then incubated in the dark for 1 min and 40 μM DCMU added, and finally assayed by fluorescence induction.

ence (Fig. 3d) of DCMU similar to intact preparations (Fig. 3, a and b). Thus, in PSII(−Ca/NaCl) preparations, there is in fact reduction of Q_B, although the shift in the redox potential of the Q_A/Q_A^- pair (150 mV [26]) caused by Ca extraction should prevent this reduction. This apparent discrepancy can be explained by the fact that the redox potential of the Q_A/Q_A^- pair, measured before in Ca-depleted PSII preparations, was obtained using EGTA [26]. Indeed, the J-P transition characteristic of Q_B reduction is absent in the PSII(−Ca/NaCl) preparations obtained using EGTA (Fig. 3e). Preillumination of PSII(−Ca/NaCl + 5 mM EGTA) preparations has virtually no effect on the F_0' yield. The results of this work demonstrate that a chelator can substantially modify PSII(−Ca/NaCl) preparations. Preillumination of OEC-depleted preparations does not form a pool of reduced plastoquinones ($F_0 = F_0'$) (Fig. 3, g and h), because electron transport in these preparation is inhibited. The addition of the electron donor, DPC, to PSII(−Mn) preparations results in the activation of electron transport (Q_B reduction) and a significant increase in the F_0 yield after preillumination (Fig. 3, i and j).

In the experiments described above, the preparations were preilluminated in the absence of DCMU. In some experiments, DCMU was added to the preparations before preillumination. This prevented the plastoquinone pool from being reduced during preillumination. The results given in Table 2 and Fig. 3b (curve *3*) demonstrate that in the absence of plastoquinone pool reduction there is an increase in the F_0' yield (by approximately 50% of maximum value). The FIK shape (Fig. 3b, curve *3*) supports the conclusion that Q_A in the preparations was indeed reduced prior to FIK measurement (there is no inflection at the O-P phase). Thus, in the presence of DCMU there is a significant decrease in the rate of Q_A^- oxidation. This fact can be explained by an increase of ~50 mV in the redox potential of the Q_A/Q_A^- pair in the presence of DCMU [47], which decreases the rate of oxidation of Q_A^-. Deceleration of Q_A^- oxidation in the presence of DCMU could also be a cause of the slow Q_A^- oxidation

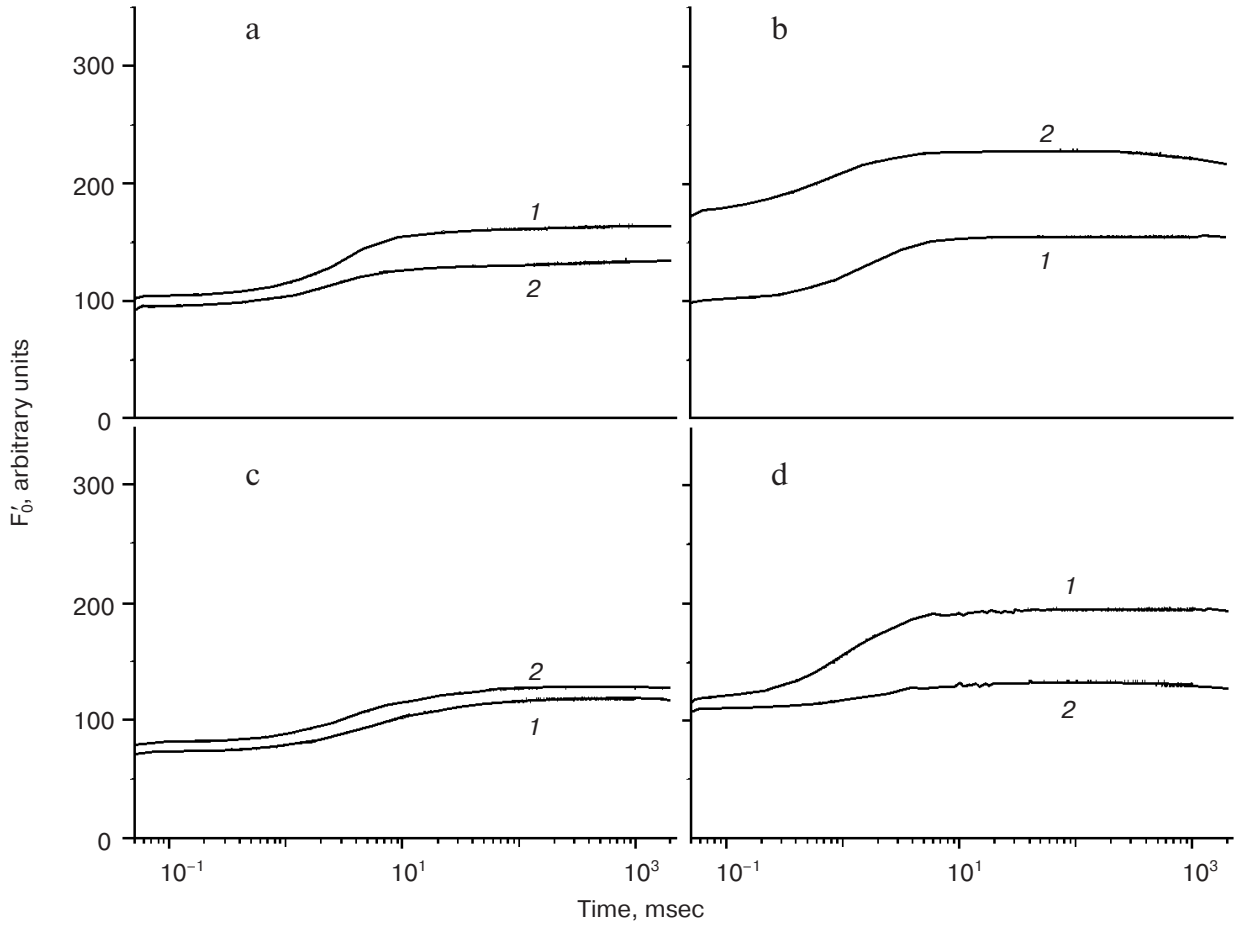

Fig. 4. Effects of metal cations on the FIK curves in intact PSII membrane preparations: a) 20 mM $Cd(NO_3)_2$; b) 100 μM $HgCl_2$, sample in buffer A; c) 100 μM $Cu(NO_3)_2$; d) 100 μM $HgCl_2$, sample in buffer A without Cl^-. FIK curves were measured in the presence of 40 μM DCMU added right before the fluorescence measurements. *1*) Kinetics of fluorescence induction in PSII preparations incubated in the dark prior to DCMU addition and the fluorescence measurement; *2*) kinetics of fluorescence induction in PSII preparations incubated in dim light for 2 min prior to DCMU addition and the fluorescence measurement.

in calcium-depleted PSII preparations containing EGTA, which was observed by Johnson et al. using EPR [28].

Thus, measurements of the F_0 yield in PSII preparations exposed to short-term preillumination with dim light reveal that F_0' in different PSII membrane preparations depends substantially on the OEC activity. Inactivation of the OEC and corresponding cessation of Mn cluster oxidation (Mn extraction) inhibits the formation of this pool. This property can be used to analyze the mechanism of inactivation of the photosynthetic activity of oxygenic organisms exposed to various factors (including environmentally dangerous factors).

In additional experiments, we studied the effects of certain heavy metal cations (Cd, Cu, and Hg) on the light-induced F_0' yield. The mechanism of the effect of the cations on PSII activity has been well established [48-51]. For example, Cd cations at millimolar concentrations were shown to expel calcium from the OEC [49]. Copper cations inhibit electron transfer at the level of Y_Z and have an effect on the acceptor side of PSII [51, 52]. Mercury cations in the absence of chloride anions inhibit the OEC [50].

The effects of various metal cations on the FIK in PSII membrane preparations measured in the presence of DCMU (added right before the fluorescence measurements) are shown in Fig. 4. The FIK curves were measured in dark-adapted PSII preparations and in PSII preparations pre-exposed to dim light for 2 min. The results of the experiments showed that Cd and Cu cations caused an insignificant decrease in the maximum fluorescence yield (compare to Fig. 3b, curve *1*), having no effect on the FIK shape. Therefore, it is impossible to draw an unambiguous conclusion about the effect of the cations on PSII. On the other hand, these cations inhibit reduction of the plastoquinone pool almost completely (low F_0' yield). This fact indicates effective inhibition on the donor side of PSII by these cations. Thus, the F_0' yield can be used as a sensitive indicator for monitoring the effects of various factors on PSII. Mercury (the neutral compound $HgCl_2$ predominates under physiological conditions [53]) does not effect the FIK or the F_0' yield (compare Fig. 4b to FIK curve shapes in PSII membranes in the presence of DCMU seen in Fig. 3b) in standard buffer solution. However, the F_0' yield decreases significantly in the absence of chloride anions (Fig. 4d). Thus, a significant difference between the FIK curve shapes before and after preillumination in the absence of chloride is evidence for a light-dependent effect of mercury.

Hence, the results of our work indicate that measurements of the light-induced F_0' yield can be used as a rapid method to study the results of the toxic effects of environmentally dangerous factors on oxygenic photosynthetic organisms.

This study was supported by the Russian Foundation for Basic Research, project Nos. 05-04-48527, 07-04-00132, 05-04-48912 (BS, LD, AB, II and AR), the United States Civilian Research and Development Foundation (BS, LD, AB, II and AR), and the Division of Energy Biosciences, Office of Science, U. S. Department of Energy (MS).

REFERENCES

1. McEvoy, J. P., and Brudvig, G. W. (2006) *Chem. Rev.*, **106**, 4455-4482.
2. Debus, R. J. (1992) *Biochim. Biophys. Acta*, **1102**, 269-352.
3. Nelson, N., and Yocum, C. F. (2006) *Annu. Rev. Plant Biol.*, **57**, 521-565.
4. Lindberg, K., and Andreasson, L. E. (1996) *Biochemistry*, **35**, 14259-14267.
5. Hoganson, C. W., and Babcock, G. T. (1997) *Science*, **277**, 1953-1956.
6. Miqyass, M., van Gorkom, H. J., and Yocum, C. F. (2007) *Photosynth. Res.*, **92**, 275-287.
7. Loll, B., Kern, J., Saenger, W., Zouni, A., and Biesiadka, J. (2005) *Nature*, **438**, 1040-1044.
8. Yano, J., Kern, J., Sauer, K., Latimer, M. J., Pushkar, Y., Biesiadka, J., Loll, B., Saenger, W., Messinger, J., Zouni, A., and Yachandra, V. K. (2006) *Science*, **314**, 821-825.
9. Latimer, M. J., DeRose, V. J., Mukerji, I., Yahandra, V. K., Sauer, K., and Klein, M. P. (1995) *Biochemistry*, **34**, 10898-10909.
10. Ghanotakis, D. F., Babckock, G. T., and Yocum, C. F. (1984) *FEBS Lett.*, **167**, 127-130.
11. Cammarata, K. V., and Cheniae, G. M. (1987) *Plant Physiol.*, **84**, 587-595.
12. Ghanotakis, D. F., Topper, J. N., Babcock, G. T., and Yocum, C. F. (1984) *FEBS Lett.*, **170**, 169-173.
13. Boussac, A., and Rutherford, A. W. (1988) *Biochemistry*, **27**, 3476-3483.
14. Boussac, A., and Rutherford, A. W. (1988) *FEBS Lett.*, **236**, 432-436.
15. Ono, T., and Inoue, Y. (1986) *Biochim. Biophys. Acta*, **850**, 380-389.
16. Boussac, A., Maison-Peteri, B., Vernotte, C., and Etienne, A.-L. (1985) *Biochim. Biophys. Acta*, **808**, 225-230.
17. Andreasson, L.-E., Vass, I., and Styring, S. (1995) *Biochim. Biophys. Acta*, **1230**, 155-164.
18. Ono, T., and Inoue, Y. (1989) *Biochim. Biophys. Acta*, **973**, 443-449.
19. Hallahan, B. J., Nugent, J. H. A., Warden, J. T., and Evans, M. C. W. (1992) *Biochemistry*, **31**, 4562-4573.
20. Boussac, A., Setif, P., and Rutherford, A. W. (1992) *Biochemistry*, **31**, 1224-1234.
21. Styring, S., Feyziyev, Y., Mamedov, F., Hillier, W., and Babcock, G. T. (2003) *Biochemistry*, **42**, 6185-6192.
22. Sivaraja, M., Tso, J., and Dismukes, G. C. (1989) *Biochemistry*, **28**, 9459-9464.
23. Boussac, A., Zimmermann, J.-L., and Rutherford, A. W. (1989) *Biochemistry*, **28**, 8984-8989.
24. Ono, T., and Inoue, Y. (1990) *Biochim. Biophys. Acta*, **1015**, 269-277.
25. Boussac, A., Zimmermann, J.-L., and Rutherford, A. W. (1990) *FEBS Lett.*, **277**, 69-74.

26. Krieger, A., Rutherford, A. W., and Johnson, G. N. (1995) *Biochim. Biophys. Acta*, **1229**, 193-201.
27. Krieger, A., Rutherford, A. W., and Johnson, G. N. (1998) *Biochim. Biophys. Acta*, **1364**, 46-54.
28. Johnson, G. N., Boussac, A., and Rutherford, A. W. (1994) *Biochim. Biophys. Acta*, **1184**, 85-92.
29. Berthold, D. A., Babcock, G. T., and Yocum, C. F. (1981) *FEBS Lett.*, **134**, 231-234.
30. Ghanotakis, D. F., and Babckock, G. T. (1983) *FEBS Lett.*, **153**, 231-234.
31. Porra, R. J., Tompson, W. A., and Kriedemann, P. E. (1989) *Biochim. Biophys. Acta*, **975**, 384-394.
32. Arnon, D. J. (1949) *Plant Physiol.*, **24**, 1-15.
33. Ono, T., and Inoue, Y. (1988) *FEBS Lett.*, **227**, 147-152.
34. Preston, C., and Seibert, M. (1991) *Biochemistry*, **30**, 9615-9624.
35. Ghirardi, M. L., Lutton, T. W., and Seibert, M. (1996) *Biochemistry*, **35**, 1820-1828.
36. Strasser, B. J. (1997) *Photosynth. Res.*, **52**, 147-155.
37. Ono, T.-A., and Inoue, Y. (1984) *FEBS Lett.*, **168**, 281-286.
38. Miyao, M., and Murata, N. (1984) *FEBS Lett.*, **168**, 118-120.
39. Miyao, M., and Murata, N. (1986) *Photosynth. Res.*, **10**, 489-496.
40. Semin, B. K., and Seibert, M. (2004) *Biochemistry*, **43**, 6772-6782.
41. Pospisil, P., and Dau, H. (2000) *Photosynth. Res.*, **65**, 41-52.
42. Pospisil, P., and Dau, H. (2002) *Biochim. Biophys. Acta*, **1554**, 94-100.
43. Heredia, P., and De Las Rivas, J. (2003) *J. Plant Physiol.*, **160**, 1499-1506.
44. Toth, S. Z., Schansker, G., and Strasser, R. J. (2005) *Biochim. Biophys. Acta*, **1708**, 275-282.
45. Strasser, R. J., Srivastava, A., and Govindjee (1995) *Photochem. Photobiol.*, **61**, 32-42.
46. Velthuys, B. R., and Amesz, J. (1974) *Biochim. Biophys. Acta*, **333**, 85-94.
47. Krieger-Lizskay, A., and Rutherford, A. W. (1998) *Biochemistry*, **37**, 17339-17344.
48. Sigfridsson, K. G. V., Bernat, G., Mamedov, F., and Styring, S. (2004) *Biochim. Biophys. Acta*, **1659**, 19-31.
49. Faller, P., Kienzler, K., and Krieger-Lindskay, A. (2005) *Biochim. Biophys. Acta*, **1706**, 158-164.
50. Bernier, M., Popovic, R., and Carpentier, R. (1993) *FEBS Lett.*, **321**, 19-23.
51. Yruella, I., Gatzen, G., Picorel, R., and Holtzwarth, A. R. (1996) *Biochemistry*, **35**, 9469-9474.
52. Jegerschold, C., Arrelano, J. B., Schroder, W. P., van Kan, P. J. M., Baron, M., and Styring, S. (1995) *Biochemistry*, **34**, 12747-12754.
53. Gutknecht, J. (1981) *J. Membr. Biol.*, **61**, 61-66.