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Heat-induced changes in the EPR signal of tyrosine D (Y_D^{OX}): a possible role of Cytochrome b559

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Abstract The present study for the first time describes a close relationship between a change in the states of Cyt b559, a damage to Mn complex and a rapid reduction of tyrosine D (Y_D) as a function of temperature in spinach thylakoid membranes. Measurements of the EPR signal of dark stable tyrosine D in heat-treated thylakoid membranes showed a gradual decay of the oxidized state of tyrosine D with the progression of temperature. Simultaneously, it leads to the conversion of high-potential Cytochrome b559 into its low-potential form. We have speculated a possible involvement of Cytochrome b559 in the primary reduction events of tyrosine D in dark at high temperature. However, rapid reduction of tyrosine D may also be due to the disassembly of the Mn clock, which causes exposure of Y_D to the lumen and thereby its reduction by some unknown factor. These conclusions are supported by the measurements of Mn^{2+} release and thermoluminescence curves of various charge pairs in heat-treated thylakoid membranes. The results reveal an important aspect on the role of Cyt b559 in PS II during temperature stress.

Keywords Photosystem II · High temperature stress · Tyrosine D · Cytochrome b559 · Electron paramagnetic resonance · Thylakoid membranes

Abbreviations

Cyt b559	Cytochrome b559
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea
FeCy	Potassium ferricyanide
Hepes	*N*-2-hydroxyethyl-piperazine-*N*,2-ethanesulphonic acid
HP	High potential
LP	Low potential
Mn^{2+}	Manganese ion
MSP	33 kDa manganese stabilizing protein
PSII	Photosystem II
TL	Thermoluminescence
Y_D^{OX}	Photo oxidized Tyrosine-160 of D2 polypeptide
OEC	Oxygen evolving complex

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Introduction

Photosystem II (PSII) is a large, multisubunit, protein complex, which is a complete structural and functional unit. The D1 and the D2 proteins of photosystem II reaction center (RC) contain two symmetrical redox active tyrosine molecules i.e. Tyrosine Z (Y_Z) and Tyrosine D (Y_D) (Zouni et al. 2001). These tyrosine molecules are oxidized by the photo-oxidized central chlorophylls P_{D1} and P_{D2}, respectively (Ferreira et al. 2004). The redox active tyrosine (Y_Z) mediates fast electron transfer between the photo-oxidized P_{680}^+ Chl moiety and the Mn cluster of PSII oxygen evolving complex (OEC). The biological function of Y_D is not well defined. The significance of Y_D for the function of PSII is, however, demonstrated by its role in the assembly of the Mn Cluster (Ananyev et al. 2002). In the normal functional OEC, oxidized Y_Z decays very fast (microseconds) and is known as signal $II_{veryfast}$ (Blankenship et al. 1975) while, its decay is comparatively slower (milliseconds) in slightly damaged PSII complex, and is called signal II_{fast} (Babcock and Sauer 1975). However, Y_D

once oxidized produces a very stable EPR signal, the decay of which takes hours (at room temperature) and weeks (at low temperature) (Kawamori et al. 1987; Vass et al. 1990). This dark stable EPR signal of the oxidized tyrosine D is known as signal II_{slow}. It has a g value of g=2.0046, a line-width of 1.5 mT, and partially resolved hyperfine peaks approx. 0.5 mT apart (Miller and Brudvig 1991).

The difference in the redox potentials and the kinetic behavior of Y_Z and Y_D is a consequence of greater polarity of the environment of Y_Z with a highly disordered hydrogen bonding and its proximity to the Mn cluster. On the contrary, the environment of Y_D is highly hydrophobic and shows a well ordered hydrogen bonding (Campbell et al. 1997; Zouni et al. 2001). The oxidation of Y_D is shown to be a pH dependent phenomenon (Babcock and Sauer 1973; Faller et al. 2002). Upon illumination of PSII membranes at cryogenic temperature (15K), a rapid oxidation of Y_D is observed at alkaline pH (8–9.5) while at lower pH (< 6.5) photo-oxidation of Y_D is completely inhibited (Faller et al. 2001). A complete oxidation of Y_D can also be achieved at room temperature by continuous illumination for about 30 seconds (Danielsson et al. 2004) at physiological pH. However, less is known about the reduction of this oxidized Y_D. There are only three components known which have the ability to reduce oxidized Y_D (Y_D^{OX}); They are the S_0 state ($t_{1/2}$ ~ 5–10 min) (Styring and Rutherford 1987), Q_A^- ($t_{1/2}$ ~ 5 days at 77K) (Kawamori et al. 1987) and the Cytochrome b559 ($t_{1/2}$ ~ 13 weeks at 203K) (Vass et al. 1990). The decay of Y_D^{OX} is reported to be biphasic where the half time of fast phase is ~ 5 min and that of the slow phase is 9–10 h. In its reduced form, Y_D causes fast reduction of the S_2 and S_3 states into subsequent lower states; however, it can not further reduce the S_1 to S_0 state. The kinetics of this decay is in the time domain of seconds (Babcock and Sauer 1973; Vass and Styring 1991; Vermaas et al. 1989; Deák et al. 1994).

The PSII complex also contains a small, membrane spanning heme-iron containing protein Cytochrome b559 (Cyt b559), transmembrane α helices of which open near the Q_B binding site at the stromal side (Loll et al. 2005). Its carboxy terminal domain is shielded by a 33 kDa manganese-stabilizing protein (MSP) at the lumenal end (Tae et al. 1988). The requirement of Cyt b559 for providing structural integrity and assembly of the functional PSII complex is well documented (Pakrasi et al. 1990, 1991). It has also been shown to protect against the donor side photo-inhibition of PS II (Poulson et al. 1995; Magnuson et al. 1999). Cyt b559 has the characteristic of occurring in different potential forms: one is the high potential (HP) form with a midpoint redox potential of 330–400 mV, and another is the low potential (LP) form having redox potential varying from 20–80 mV (Kaminskaya et al. 2005). It is well known that in normal thylakoids, most (80%) of the Cyt b559 occurs in the high potential (HP) state while about 20% remains in the low potential (LP) state (Mizusawa et al. 1995). However, the inactivation treatments of OEC such as Tris washing (Ghanotakis et al. 1986), mild heat stress (Cramer et al. 1981), low temperature (Vass et al. 1990) and low pH (Pospišil et al. 2006) etc. leads to the conversion of the HP form of Cyt b559 to the LP Cyt b559 form. Under mild heat stress (37°C), the HP to LP conversion of Cyt b559 is faster and the extent of oxidation-reduction changes increase (Canaani and Havaux 1990). It has also been proposed that in PSII particles, in heat-treated chloroplasts and in trypsin-digested chloroplasts, HP Cyt b559 is absent (Horton et al. 1978). Furthermore, It has been shown that heat treatment to thylakoid membranes increase the likelihood of trypsin digestion of the NH_2-terminal domain of α-chain of Cyt b559 (Jang and Tae 1996) possibly due to the removal of the manganese-stabilizing protein (MSP) (Nash et al. 1985; Enami et al. 1994).

Heating induces a gradual inhibition of the donor side of PS II, which leads to the increase in the life time of P_{680}^+ and thus increases the probability of donation of the electron from Y_D to P_{680}^+ (Buser et al., 1990). Reduced Cyt b559 can be oxidized by P_{680}^+ through a sequential or branched pathway involving a carotenoid and a special chlorophyll denoted Chl_z (Faller et al., 2001). The carotenoid-Chl_z pathway becomes operative only when P_{680}^+ becomes abnormally long lived e.g. in the case of disorganization of the Mn cluster. An activation of the cyclic electron flow around PS II involving Cyt b559 under heat stress is predicted (Schreiber and Neubauer 1990). It is suggested that the cyclic electron transfer around PS II could play an essential role in the substitution of the thermally damaged OEC by donation of electron from the PS II acceptor side to P_{680}^+ or Yz^{OX} preserving the photochemical activity of PS II at higher temperatures (Havaux 1998). Involvement of Y_D in PS II-mediated electron transfer in green alga *Chlamydobotrys stellata* grown under photo-heterotrophic conditions where the oxygen evolution and $NADP^+$ reduction are drastically diminished has been shown (Mende et al. 1996). The role of Cyt b559 and Y_D in protection against photoinhibition of PS II was suggested (Magnuson et al. 1999). On heating, loss of the oxygen-evolution activity and the reduction of Y_D^{OX} was shown to occur simultaneously (K_1=1.0 min^{-1}) and was correlated to the Ca^{2+}-release (Pospišil et al. 2003).

In the present study, we have established a close relationship between the rapid reduction of Y_D^{OX}, the HP to LP conversion of Cyt b559, and the disorganization of the Mn clock as a function of temperature. It is inferred from the available literature that the primary targets of heat-induced damages are structurally linked to Cyt b559 (Loll et al. 2005; Tae et al. 1988; Cramer et al. 1981; Canaani and Havaux 1990; Horton et al. 1978; Jang and Tae 1996).

Our results suggest redox states of Y_D to be an indicator of PSII damage during temperature stress. The potential ability of these cofactors to accept/donate electrons during temperature stress seems to rescue PS II against the oxidative damage.

Materials and methods

Preparation of thylakoid membranes

All the procedure of isolation and washing were undertaken under dim green light at 4°C unless stated otherwise. The thylakoid membranes were prepared from dark-adapted spinach leaves, which were plucked from the field in the evening and deveined after thorough washing with ice-cold distilled water and kept overnight at 4°C. The isolation methods were followed as described in (Kuwabara and Murata 1982). The isolated membranes were suspended in a storage media containing 50 mM tricine, 10 mM NaCl (pH 7.6) and 50% (v/v) glycerol, and stored in liquid nitrogen in dark until further use. Prior to the experiment, the thylakoids membranes were slowly thawed at 0–4°C and then washed once at 7500×g for 5 min at 4°C, using 20:1 ml ratio of washing medium (20 mM NaCl and 50 mM Hepes, pH −7.6). The pellet was resuspended in the suspending medium that contained 0.35 M sucrose, 1 mM NaCl, 1 mM $MgCl_2$ and 50 mM Hepes (pH 7.6) and was subjected to heat treatment as described in (Tiwari et al. 2007). Chl concentration was determined by the method described in (Porra et al. 1989).

Heat treatment

The thylakoids were subjected to heat treatment in dark for 5 min at 35°C, 40°C, 42°C and 45°C in a water bath (Julabo F-10) with continuous gentle shaking. After the heat treatment the sample tubes were immediately transferred to the ice bath and kept on ice until assay.

EPR measurements

The EPR spectra were recorded on a Jeol X-band EPR spectrometer, equipped with a TM mode variable temperature cavity having DVT4 controller. The samples were filled in calibrated EPR micropipettes and measurements were done at room temperature and at various temperatures in dark as well as with continuous illumination by a 250W projector lamp. The post-illuminated spectra of these 60 seconds illuminated samples were recorded in dark, to record the maximum amplitude of the oxidized Y_D^{OX}.

Cyt b559 measurements

The amount of different redox states of Cyt b559 was estimated spectrophotometrically in DCMU-treated thylakoid membranes using a Shimadzu UV-2400 PC spectrophotometer as described in (Lazar et al. 2005). Unheated and heat-treated thylakoid were diluted to 50 μg Chl ml^{-1} immediately before the Cyt measurements. Step by step, diluted thylakoids in the test cuvette and the reference cuvette were treated with DCMU 20 μM (both cuvettes), ferricyanide 20 μM (reference cuvette), hydroquinone 4 mM (test cuvette) and sodium ascorbate 5 mM (test cuvette). Each step was followed by 6 min dark adaptation and spectra were recorded from 520 nm to 580 nm. The spectra of each of the samples were recorded five times. The amount of different states of Cyt *b*559 was calculated from average spectra of five measurements.

Thermoluminescence measurements

Thermoluminescence glow curves were measured with a new high sensitivity TL instrument developed at the Centre for Advanced Technology, Indore as described in (Bhatnagar et al. 2002). A 10 μl thylakoid suspension having Chl concentration equivalent to 2 mg Chl ml^{-1} was spread on a 0.45 μ nitrocellulose filter disc of 1 cm diameter. This was kept on a 0.02 mm thick aluminum foil and was covered with a quartz filter. Before starting the cooling–heating cycle, the sample was incubated for two min at 20°C in dark. The sample was illuminated with a white light LED at −20°C for 30 seconds and after completion of the exposure it was cooled to −60°C with a cooling rate of 0.8°C/s followed by the same heating rate up to +60°C. The luminescence data were recorded at 5 points per second simultaneously with the time and temperature recording. Minimum five TL curves were measured for each of the treatments.

Results and discussion

Temperature-dependent fast reduction of dark-stable Y_D^{OX}

The stored thylakoid membranes (~7–10 weeks, at 77 K) were used to monitor the temperature-dependent changes in the Y_D^{OX} in dark. In these thylakoid membranes 70% of the Y_D was present in the oxidized form (Y_D^{OX}) as compared with the amplitude of Y_D^{OX} signal after illumination (Fig. 1). In the dark-adapted thylakoid membranes, when temperature treatment is given, the Y_D^{OX} gets converted to Y_D as indicated by the reduced amplitude of Y_D^{OX} signal (marked by an arrow in Fig 1). In 45°C-treated thylakoid membranes, <2% of the Y_D remained in the oxidized state (Fig. 1) suggesting thereby that the Y_D^{OX} got electron from

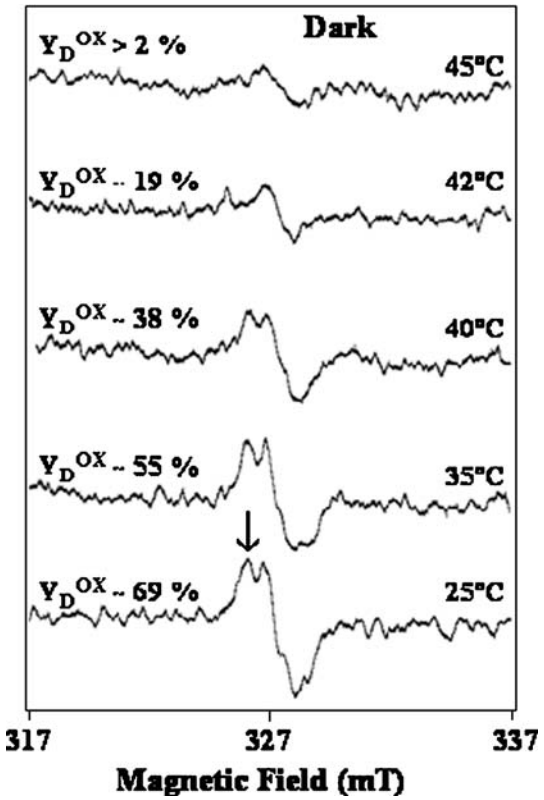

Fig. 1 EPR measurements of dark stable signal II_{slow} of Y_D^{OX} (in dark) in untreated and 5 min heat-treated spinach thylakoid membranes. Experimental condition were as follows: frequency 9.441 GHz; center field 327 mT; sweep width 7.5 mT; microwave power 2 mW; field modulation 100 kHz. The amount of Y_D^{OX} was calculated by comparison with the signal of post-illuminated spectra where Y_D was fully oxidized

somewhere even in the dark when temperature stress was given to the thylakoid membranes. We used long dark-adapted (~7–10 weeks old) thylakoid membranes and thus the possibility of the presence of the S_0-state and Q_A^- is negligible in our samples. In such cases, majority of the centers were present in the $S_1Y_D^{OX}$ state (Styring and Rutherford 1987) and thus the possibility of recombination of Y_D^{OX} with the S_0 state and Q_A^- could be safely ruled out.

Heat-induced changes in Cyt b559

We measured the amount of the HP (oxidized) and the LP (reduced) forms of Cyt b559 in dark in control and in heat-treated PS II membranes. Results show an interesting correlation between the stoichiometry of the reduction of Y_D^{OX} and the conversion of Cyt b559 from HP→LP form (Fig. 2). This suggested that Y_D^{OX} was probably reduced owing to the conversion of HP→LP form of Cyt b559. However, as seen in Fig. 2, heat treatment resulted in the oxidation of ~80% of the HP form of Cyt b559 whereas reduction of Y_D^{OX} was found to be as high as 98%. This suggested that the reduction of the oxidized Y_D was not solely depended upon the conversion of Cyt b559 from HP to LP form. It might be additionally reduced as a result of reaction with some unknown reductant(s) of the lumen.

Heat-induced damage to Mn complex

The oxygen evolution complex is a cubane like Mn_3CaO_4 cluster with a mono-μ-oxo bridge to four Mn ions (Ferreira et al. 2004), where the Mn ions with its four oxidation states play an essential function during photolysis of water (Kok et al. 1970). Recently, in heat-treated thylakoid membranes we have shown that the release of Mn^{2+} is not always accompanied with the loss of oxygen evolution activity (Pospišil et al. 2003; Tiwari et al. 2007). We observed that in 45°C-treated thylakoid membranes the maximum release of Mn^{2+} was only 22% that was fairly less than the loss of oxygen evolution activity at the same temperature (90% lost). Although the heat treatment did not cause very high Mn^{2+} release up to 45°C, yet it indicates some perturbation in the polypeptides of the OEC that could possibly result in an increase in the accessibility of Y_D to the lumen and thereby causing a complete reduction of the remaining Y_D^{OX}. A recent study suggests that at high temperature (47°C) release of 18 kDa protein is the main cause behind the loss of essential Ca ion from the Mn_4Ca complex (Pospišil et al. 2003; Barra et al. 2005). However, a slight change in the conformation of the bound 18 kDa may also facilitate the access of Y_D from the bulk and may favor a rapid reduction of it in dark at comparatively lower temperatures (35 to 45°C). Our conclusions are further substantiated by the thermoluminescence (TL) measurements of heat-treated thylakoid membranes. The thermolu-

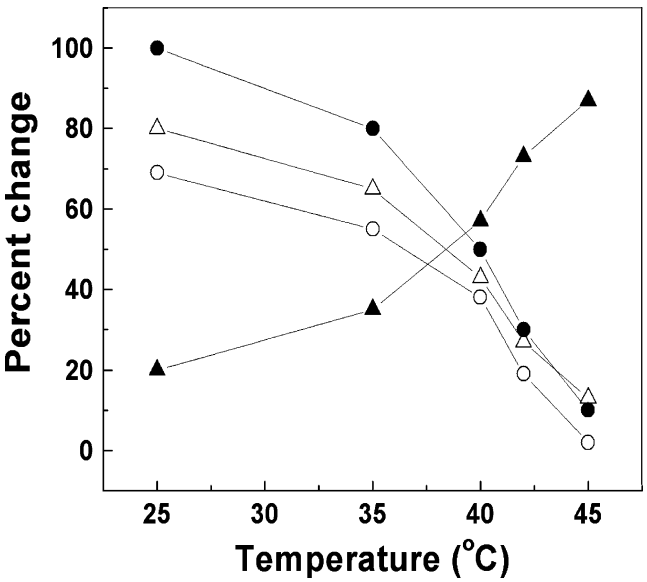

Fig. 2 Relative percent change in the amounts of Y_D^{OX} (○), HP Cyt $b559^{red}$ (△), LP Cyt $b559^{OX}$ (▲) and oxygen evolution process (●) as a function of temperature

minescence glow curves are good indicators of charge recombination between the 'S-states' of oxygen clock and Q_A^-/Q_B^- of the PSII acceptors (Sane and Rutherford 1989). Three TL bands distinctly appeared in unheated thylakoid membranes *i.e.* the Q band (10°C), the B band (between 20–30°C) and the C band (40–50°C) (Fig. 3). The B band is assigned to the recombination of Q_B^- with the S_2 or S_3 states (Rutherford et al. 1982, 1985; Demeter and Vass 1984); the Q band and the C band are assigned to the recombination of Q_A^- with the S_2 state and Y_D^{OX}, respectively (Rutherford et al. 1982; Demeter and Vass 1984; Johnson et al. 1994; Krieger et al. 1993; Rutherford et al. 1984). As evident from Fig. 3, the Q and the C bands disappeared at 35°C while the B band decreased gradually with the increasing temperature and finally vanished at 45°C. This indicates a progressive loss in the advancement of the S-states due to the thermal inactivation of the Mn complex. Relatively fast decay of the C band in heat-treated thylakoid membranes corresponds with the disappearance of the EPR signal arising from Y_D^{OX}.

Effects of high temperature on the re-oxidation of tyrosine D

After the measurement of the EPR signal of Y_D^{OX} in dark, the heat-treated samples were continuously illuminated for 30 s and the EPR signal was recorded. The experiments were done in two ways: i) The EPR signals were measured at 25°C (Fig. 4a), ii) The EPR signals were measured at the respective temperature of heat treatment (Fig. 4b). In

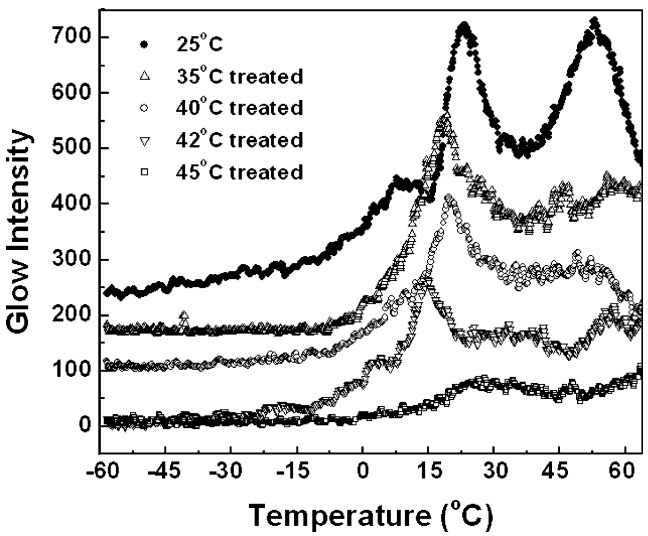

Fig. 3 TL glow curves of pre-heated control (25°C) and various heat-treated thylakoid membranes. 10 µl thylakoid suspension having Chl concentration equivalent to 2 mg Chl ml^{-1} was subjected to a single cooling and heating cycle from +25°C to −60°C and −60°C to +60°C, respectively with a cooling rate of 0.8°C/s. During cooling cycle samples were illuminated at −20°C for 30 s with white LED. Luminescence data were recorded at 5 points per second simultaneously with time and temperature recording

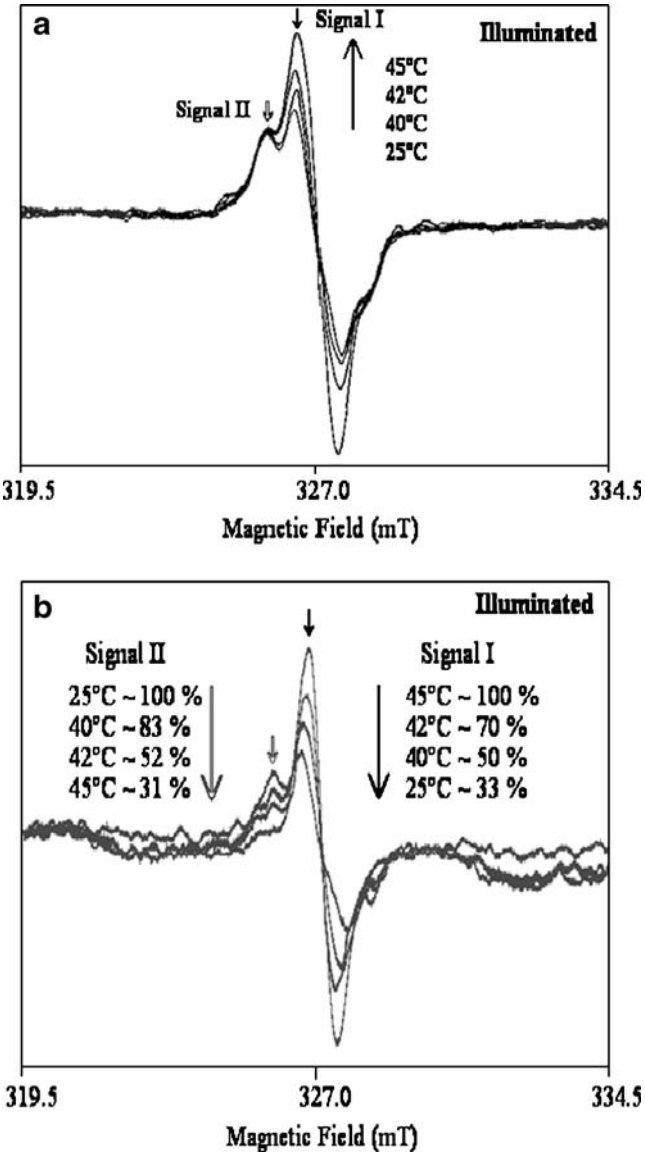

Fig. 4 EPR spectra of heat-treated and untreated thylakoid membranes during illumination, (**a**) measured at 25°C; (**b**) measured at the temperature of pre-treatment. Experimental conditions were the same as described in the legend of Fig. 1

DCMU-treated thylakoid membranes, illumination generates maximum amplitude of the EPR signal at g=2.0025 (Signal I) which corresponds to the photo-oxidized P700 ($P700^+$), while Signal II at g=2.0046 corresponds to the EPR signal of Y_D^+. As seen in Fig. 4a, the extent of re-oxidation of Y_D was the same in heat-treated thylakoids and in control suggesting that 100% Y_D^{OX} was formed in untreated and heat-treated thylakoid membranes and no damage occurred to the photo-oxidation process of Y_D. However, the photo-oxidation of Y_D was significantly inhibited when the heat-treated samples were illuminated at respective temperatures (Fig. 4b). This suggests that at high temperature, either P_{680}^+ is not able to draw electron from Y_D or rapid reduction of photo-oxidized Y_D is taking

place at such a fast rates that no stable Y_D^{OX} signal is detectable at this temperature. This conclusion was supported by the fact that when heat-treated samples were brought back at 25°C; the stable signal of Y_D^{OX} reappeared after illumination. However, no change in the HP and LP states of Cyt b559 was noticed after illuminating the heat-treated samples. Similarly oxygen evolution was also not observed in heat-treated thylakoid membranes. Our samples were measured at pH 7.6 and chances of effective electron donation from Y_D to P_{680}^+ are fairly good. In our samples, the fluorescence kinetics of heat-treated samples shows that Fv decreased as a function of increasing temperature, and diminished completely above 45°C (data not shown). It may be because of the accumulation of less fluorescent state $P_{680}^+Q_A$ (open center) at high temperature causing a decline in the intensities of the Q and the C thermoluminescence bands due to the unavailability of Q_A^-. The enhanced photo-oxidized P_{700}^+ accumulation indicates that electron from Q_A^- is also not going towards P_{700}. In such a case there is a possibility for this electron to undergo cyclic electron flow within the PSII complex ($Y_D \rightarrow P_{680}^+ \rightarrow$ ...?) as suggested earlier (Schreiber and Neubauer 1990; Kouril et al. 2004). The possibility of Carotene-Chl_ZD_2-Y_D pathway (Hanley et al. 1999) in this process can also not be ruled out.

Effects of high temperature on re-reduction kinetics of tyrosine D

The re-reduction of Y_D^{OX} was studied in untreated and heat-treated thylakoid membranes by illuminating the samples for 30 s at room temperature and then incubating them in dark for 10 min. Upon illumination, we get EPR signal from both Y_z^{OX} and Y_D^{OX}. However, both the signals differ in their decay properties. In normal thylakoid membranes Y_Z decays very fast while the reduction of Y_D^{OX} ($Y_D^{OX} \rightarrow Y_D$) takes several minutes to hours. As seen in Fig. 5, there was an insignificant reduction of Y_D^{OX} in the control samples. However, decay/reduction of Y_D^{OX} in heat-treated thylakoid membranes was slightly faster. This further supports our contention that at high temperatures, due to membrane perturbation and/or damage to the Mn complex, the Y_D gets slightly exposed to the lumen resulting in its faster reduction in heat-treated thylakoid membranes.

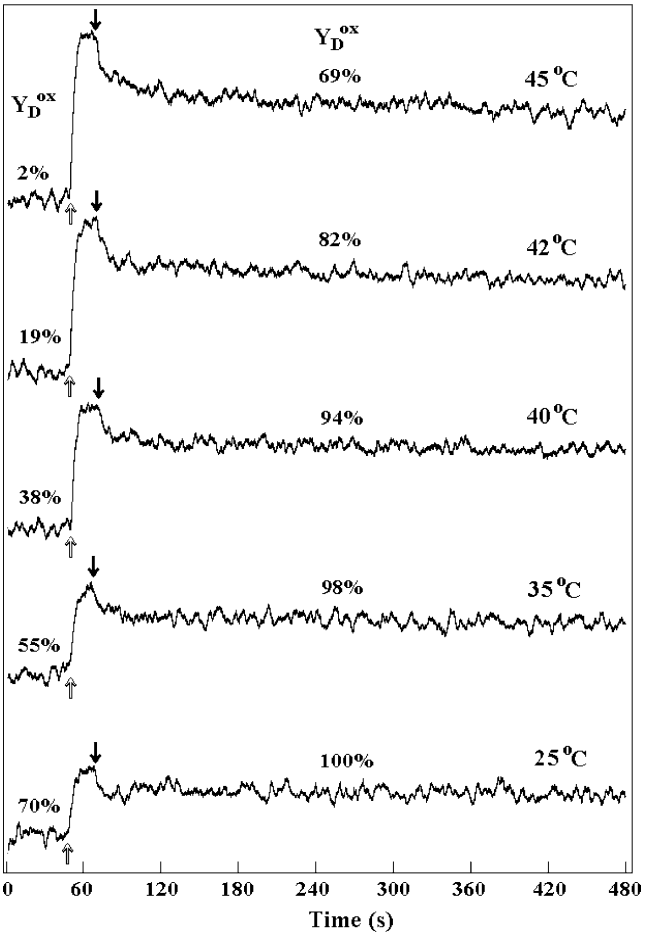

Fig. 5 EPR traces of formation and decay kinetics of Y_D^{OX} at room temperature in control and heat-treated thylakoid membranes. The amount of Y_D^{OX} was obtained by relative change in amplitude in comparison to the post-illuminated spectra of complete Y_D^{OX}. Open and closed arrow represents turning on and off of light, respectively. Experimental conditions were the same as described in the legend of Fig. 1 except that sweep width kept zero and center field was adjusted on the position of occurrence of high field peak of Y_D^{OX}

Conclusions

The temperature dependence of the reduction of Y_D^{OX}, change in the states of Cyt b559, and damage to the oxygen evolution activity in terms of release of manganese and loss of progression of the S-states are described. The temporal separation of all these events are highly complicated. Pospišil et al. (2003) have demonstrated in a temperature-jump study that heat-induced decrease in the Y_D^{OX} EPR signal is related to the first phase of heat-induced disassembly of the water-splitting complex that involves release of Ca^{2+}. However, we here suggest a possible involvement of Cyt b559 in the primary reduction events of Y_D^{OX} and later in the disassembly of the Mn complex. We have shown here that the kinetics of loss of oxygen evolution or the S-states cycle is similar to that of the reduction kinetics of Y_D^{OX}. It starts from 35°C onwards and is totally diminished in 45°C-treated thylakoid membranes. However, the maximum release of Mn is found to be 22–30% in 45°C-treated samples. Similarly, the Q ($Q_A^-S_2$) and the B ($Q_B^-S_2/S_3$) bands of TL glow curves are completely inhibited above 42°C, which further suggests the loss of formation of charge pairs in which the higher S-states are involved. Above this temperature release of 18, 23 and

33 kDa proteins is also reported (Enami et al. 1994; Yamane et al. 1998; Barra et al. 2005) which could be the second most plausible reason for increased accessibility of Y_D to the lumen.

Current study also gives indications of the association of Cyt b559 with heat-inactivation events. The definite role of Cyt b559 and the mechanism involved in heat-induced damage needs to be further confirmed by a detailed study involving potentiometric titrations and EPR measurements.

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