Photosystem II in Different Parts of the Thylakoid Membrane: A Functional Comparison between Different Domains†

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ABSTRACT: The electron transport properties of photosystem II (PSII) from five different domains of the thylakoid membrane were analyzed by flash-induced fluorescence kinetics. These domains are the entire grana, the grana core, the margins from the grana, the stroma lamellae, and the Y100 fraction (which represent more purified stroma lamellae). The two first fractions originate from appressed grana membranes and have PSII with a high proportion of O_2-evolving centers (80−90%) and efficient electron transport on the acceptor side. About 30% of the granal PSII centers were found in the margin fraction. Two-thirds of those PSII centers evolve O_2, but the electron transfer on the acceptor side is slowed. PSII from the stroma lamellae was less active. The fraction containing the entire stroma has only 43% O_2-evolving PSII centers and slow electron transfer on the acceptor side. In contrast, PSII centers of the Y100 fraction show no O_2 evolution and were unable to reduce Q_B. Flash-induced fluorescence decay measurements in the presence of DCMU give information about the integrity of the donor side of PSII. We were able to distinguish between PSII centers with a functional Mn cluster and without any Mn cluster, and PSII centers which undergo photoactivation and have a partially assembled Mn cluster. From this analysis, we propose the existence of a PSII activity gradient in the thylakoid membrane. The gradient is directed from the stroma lamellae, where the Mn cluster is absent or inactive, via the margins where photoactivation accelerates, to the grana core domain where PSII is fully photoactivated. The photoactivation process correlates to the PSII diffusion along the membrane and is initiated in the stroma lamellae while the final steps take place in the appressed regions of the grana core. The margin domain is seemingly very important in this process.

The photosynthetic membrane, the thylakoid, has a very complex structure. In higher plants it is differentiated into at least three domains with different composition and function (Figure 1). These domains are the following: the stroma lamellae which are single paired and exposed to the chloroplast stroma, the grana margins which are also exposed to the stroma and form an annulus around the third domain, the stacked membranes of the grana core (*1*). The end membranes of a grana stack may constitute a fourth domain (*2*).

In many preparations, the thylakoid membrane is solubilized with detergents. This might have consequences for the functional integrity of the photosynthetic complexes and their interaction which involves the lipid- and water-soluble electron carriers plastoquinone and plastocyanin. However, detergents can be avoided and the three domains can be isolated in the form of vesicles, by fragmentation using mechanical press treatment or mild sonication followed by centrifugation and separation with aqueous polymer two-phase systems (*3*, *4*). Such fractionation studies have shown that photosystem I (PSI)[1] and photosystem II (PSII) are segregated in the thylakoid membrane. There is more PSII in the stacked membranes while PSI is predominantly found in the stroma region. The margins have a more mixed composition (*5*).

There is also heterogeneity in both photosystems with respect to their Chl antenna size and electron transfer properties. PSIIα is located in the grana core while PSIIβ is located in the stroma lamellae together with PSIβ (both α systems have a larger antenna size than the respective β systems). PSIα was found in the margins of the grana. The distribution of the two photosystems and other components in the thylakoid membrane is described elsewhere (*4*, *6*, *7*).

PSII centers are heterogeneous and vary in several important aspects (reviewed in *8*−*11*). PSII initiates the electron transport chain by coupling water oxidation with

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[1] Abbreviations: Chl, chlorophyll; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCIP, 2,6-dichlorophenolindophenol; DPC, 2,2′-diphenylcarbonic dihydrazide; F_0, initial level of fluorescence; F_V, variable fluorescence; PpBQ, phenyl-*p*-benzoquinone; PQ, plastoquinone; PSI, PSII, photosystems I and II, respectively; Q_A, Q_B, primary and secondary quinone acceptors in PSII; Y_Z, secondary electron donor in PSII, Tyr161 on the D1 protein; Y_D, additional secondary donor in PSII, Tyr161 on the D2 protein; BS, subfraction of the thylakoid membrane originating from the core of grana-appressed membranes; B3, from the entire grana lamellae; T3, from the stroma lamellae; Y100, probably further purified stroma lamellae.

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the reduction of plastoquinone (*12, 13*). One can distinguish between PSII units having a different antenna size (PSIIα centers have a bigger Chl antenna than PSIIβ centers) and PSII units differing in their redox properties (water oxidation and Q_B-reducing properties). A fraction of PSII is unable to reduce the second plastoquinone acceptor, Q_B. These centers are known as non-Q_B-reducing centers (*11*). The PSII complexes most active in water oxidation are Q_B-reducing, PSIIα centers. These are located in the grana stacks, while non-Q_B-reducing centers of the PSIIβ type are located in the stroma lamellae (see *11* for a discussion). In one model (*9, 14*), all three types of static heterogeneity, i.e., membrane location (in the appressed grana or in the nonappressed stroma lamellae), light harvesting efficiency (PSIIα or PSIIβ), and electron transfer activity (Q_B-reducing or non-Q_B-reducing centers), represent the same active and nonactive PSII complexes.

Most studies of functional heterogeneity of PSII have focused on analysis of either grana stack preparations or some preparation of stroma lamellae. However, there are very few comparative studies of PSII in the different subcompartments of the thylakoid membrane. Furthermore, little is known about PSII in the margins and in the different parts of the stroma lamellae. Here, we analyze PSII in five compartments of the thylakoid membrane (Figure 1), and we are able to compare the integrity of PSII between those subfractions. It is essential for this kind of comparative investigation that the preparations are derived under mild conditions avoiding detergent treatments. We have prepared our fractions with well-adjusted noninvasive sonication and phase fractionation techniques (*3, 4*). We have then performed detailed analysis of PSII activity using flash-induced variable fluorescence measurements.

The decay kinetics of flash-induced variable Chl *a* fluorescence are a useful and informative tool to study electron transfer reactions in PSII (*15*−*18*). In dark-adapted samples, the primary quinone acceptor Q_A is oxidized, and the fluorescence is low (F_0 level, open centers, *19*). A short flash forms Q_A^- (closed centers) which results in a rapid rise of the variable Chl *a* fluorescence (F_V). The decay of the variable fluorescence, which then occurs, consists of several kinetic phases and reflects the intactness and status of the PSII centers. Decay of the flash-induced fluorescence due to forward electron transfer from Q_A^- to oxidized Q_B occurs with $t_{1/2} = 100-250$ μs, while the reduction of the semiquinone Q_B^- occurs in 300−600 μs (*17, 18*). If the Q_B site is not occupied, forward electron transfer from Q_A^- is limited by Q_B binding which normally can be observed as a decay of F_V in 2−10 ms (*18*). In PSII centers lacking the Mn cluster (for example, prior to photoactivation), the forward electron transfer from Q_A^- is even slower, and the fluorescence decays in 30−40 ms (*20*). A very slow phase (usually several hundred milliseconds to 1 or 2 s) is due to recombination between Q_A^- and the S_2 state of the O_2-evolving complex and occurs in O_2-evolving centers which are not able to reduce Q_B for one reason or another. In the presence of DCMU, the recombination between Q_A^- and the donor side of PSII dominates the fluorescence decay, making it possible to analyze the state of the donor side of PSII (*15, 20*−*22*). Thus, flash-induced fluorescence decay measurements can provide information about the functional status of both the donor and the acceptor sides of PSII.

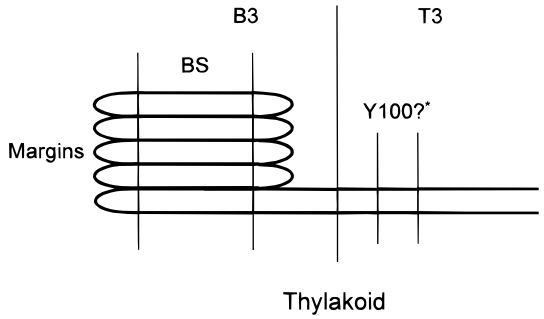

FIGURE 1: Schematic representation of the terminology and the different domains of the thylakoid membranes studied in this work. The BS, B3, and T3 fractions are also denoted the grana core, the grana, and the stroma lamellae, respectively. Asterisk: The exact origin of the Y100 fraction within the stroma-exposed region is unknown.

In this study, we describe the electron transport in PSII in vesicle preparations from the grana core, the grana margins, and the stroma. The results demonstrate large differences in fluorescence behavior between the different pools of PSII and increase further our understanding of the heterogeneity of PSII in different compartments of the thylakoid membrane.

MATERIALS AND METHODS

Preparation and Fragmentation of Thylakoid Membranes. Spinach (*Spinacia oleracia L.*) was grown at 20 °C with a light/dark period of 12 h and a light intensity of 300 μE m^{-2} s^{-1}. The spinach leaves were dark-adapted 24 h prior to chloroplast isolation. The spinach chloroplasts were isolated and osmotically broken, and the thylakoid membranes were obtained for further fragmentation as described earlier (*23*).

The different preparations (Figure 1) were obtained from the thylakoid membranes by sonication and centrifugation. Detergents were avoided in all steps. The stroma lamellae membrane fraction (T3) and the grana lamellae membrane fraction (B3) were obtained according to (*23*). The stroma thylakoid vesicles (Y100) were prepared essentially as in (*24*). The grana margins were isolated as described in (*5*), and the grana core vesicles (BS) were prepared as in (*25*). All fractions were resuspended in 100 mM sucrose, 5 mM NaCl, and 10 mM phosphate buffer (pH 7.4) at a concentration of 2−3 mg of Chl mL^{-1} and stored at 77 K.

Electron Transport Measurements. The O_2 evolution was measured with a Hansatech Clark-type O_2 electrode at 20 °C using saturating light filtered through a OG590 Schott filter at a sample concentration of 10 μg of Chl mL^{-1} with 0.5 mM PpBQ as artificial electron acceptor. Electron transfer from H_2O was also measured as DCIP photoreduction at 560 nm in a Shimadzu UV3000 spectrophotometer equipped with sideways illumination. The assay was performed at a sample concentration of 10 μg of Chl mL^{-1} in the presence of 10 μM DBMIB, 6 μg/mL gramicidin, and with or without 500 μM DPC as external electron donor (DPC was added from fresh 25 mM stock solutions in ethanol). The DCIP concentration was 35 μM. The electron transport activity was calculated from the linear phase, within the first 30 s of illumination. The fraction of O_2-evolving PSII centers was estimated as the ratio between the electron transfer from water to DCIP (the O_2-evolving PSII centers) and the electron

Table 1: PSII Electron Transfer Activity in Different Domains of the Thylakoid Membranes from Spinach

domain of thylakoid membrane (Figure 1)	O_2 evolution [μmol of O_2 (mg of Chl)$^{-1}$ h^{-1}]	DPC-mediated DCIP reduction [μmol of $DCIP_{red}$ (mg of Chl)$^{-1}$ h^{-1}][a]	O_2-evolving centers (% of total centers, V_{H_2O}/V_{DPC})[b]	F_V/F_0	Chl *a*/*b* (mol/mol)[c]	no. of preparations analyzed
thylakoids	120	45	80	0.70	2.9	5
grana core (BS)	250−300[c]	64	91	1.10	1.8−2.0	3
grana (B3)	200−250[c]	55	84	0.96	2.2−2.4	4
margins	102	37	66	0.48	3.0−3.3	3
stroma (T3)	80	22	43	0.27	4.5−5.0	4
Y100	0	6.7	0	0.20	6.0−6.7	3

[a] Measured only in the presence of exogenous electron donor DPC. [b] Estimated from the DCIP reduction measured in the absence and presence of the DPC (see footnote 2). [c] The numbers reflect the variability between preparations.

transfer to DCIP measured in the presence of the exogenous donor DPC (*26*).

Flash-Induced Fluorescence Measurements. Chlorophyll *a* fluorescence was detected with a PAM100, pulse amplitude modulated fluorometer (Walz, Effeltrich, Germany). The monitoring light pulses from a light emitting diode were provided at 650 nm and with 1 μs duration at a frequency of 1.6 kHz, except as noted. Single saturating actinic flashes of approximately 8 μs width at half-maximum were provided by a Walz XST-103 xenon flash lamp. The fluorescence detection started 120 μs after the flash. Data were collected and analyzed using the Q_A-FIP program (version 4.3, Q_A-Data Oy, Turku, Finland). The sample concentration was 20 μg of Chl mL^{-1}. Each sample was dark-adapted for 1 min before fluorescence detection. The multiexponential least-squares curve-fitting was done using the Levenberg−Marquardt algorithm and free running parameters according to

$$F(t) = F_0 + A_1 e^{-k_1 t} + A_2 e^{-k_2 t} + ... + A_n e^{-k_n t}$$

where $F(t)$ is the fluorescence value at time t, A_n is the amplitude of the different decay phases, k_n are rate constants, and F_0 is the initial fluorescence before the actinic flash. It should be noted that the F_V/F_0 (Table 1) induced by a single turnover flash was always much lower than the F_V/F_0 induced by continuous illumination (not shown).

RESULTS

Characterization of the Photosystem II Activity. The aqueous two-phase separation technique in combination with sonication (*3*, *4*) allows separation, with remarkable yield, of different domains of the thylakoid membrane without the use of detergents (Figure 1). We have compared the function of PSII in the appressed (grana or B3) and nonappressed (stroma or T3) fractions of the thylakoid membrane. In addition, we have separated the grana core (BS) and the margin domain (Figure 1) and studied them independently. We have also purified the nonappressed membranes further to obtain the Y100 fraction which is completely devoid of any granal contamination (*24*, *27*)

It was previously found that these fractions are different in the content of the PSI, PSII, and cytochrome *b*/*f* complexes (*4*, *6*, *7*). This is confirmed here. In addition, all fractions were different in PSII activity. The most efficient O_2-evolving PSII centers are found in the BS and B3 fractions, and the O_2 evolution varies between 200 and 300 μmol of O_2 (mg of Chl)$^{-1}$ h^{-1}. The T3 fraction showed 3−4 times lower O_2-evolving activity on a Chl basis (Table 1) while the Y100 fraction showed no O_2 evolution at all. The activity in the margin fraction was 102 μmol of O_2 (mg of Chl)$^{-1}$ h^{-1} (Table 1).

We also tested whether the fraction of O_2-evolving PSII centers varied between the subfractions. The percentage of O_2-evolving centers can be determined from the V_{H_2O}/V_{DPC} ratio[2] (Table 1). About 80% of the PSII centers in the intact, entire thylakoid were active in O_2 evolution. The BS fraction from the appressed grana core has the highest fraction of O_2-evolving centers: 91%. In the margins about 66% and in the T3 about 40% of the PSII centers are O_2-evolving. No O_2-evolving centers were found in the Y100 fraction although they contained a small fraction of PSII as judged from the estimation of DPC-mediated DCIP reduction (V_{DPC}) and variable fluorescence (see below).

The grana fraction (B3), which is composed of membranes from both the appressed grana core and the margins, has, as expected, a fraction of O_2-evolving centers (84%) intermediate between the purified margin (66%) and grana core (BS) fractions (91%, Table 1). Interestingly, these numbers can also be used to calculate the relative fraction of PSII centers in the margins and the grana core regions, and our results suggest that about 30% of PSII in the grana (B3) fraction is found in the margins. Consequently, 70% of the PSII centers are found in the grana core.

The variable Chl *a* fluorescence, F_V, is a sensitive indicator of the PSII activity (*19*, *28*). The photochemical yield induced by a single flash, here expressed as F_V/F_0, was unevenly distributed between the different domains (Table 1). The highest ratios, indicative of a large fraction of highly functional PSII, were found in the granal part of membrane: 1.10 for the grana core (BS) and 0.96 for the entire grana stack (B3) fractions, respectively. The lowest ratio was found in the Y100 and stroma lamellae (T3) fractions—0.20 and 0.27, respectively—suggesting that PSII, although present, was essentially inactive in electron transport. This is in agreement with the low O_2 evolution in those fractions. The margins show a F_V/F_0 ratio = 0.48, intermediate between the granal and stromal fractions.

Thus, we found most of the active PSII in the appressed regions. No PSII centers in the purified stroma fraction Y100

[2] V_{H_2O} is the reduction of DCIP when only water is available as electron donor and measures O_2-evolving centers. V_{DPC} is measured in the presence of DPC which is a potent donor to PSII in the absence of a functional Mn cluster. V_{DPC} consequently measures all PSII centers, both O_2-evolving and centers without the Mn cluster. V_{H_2O}/V_{DPC} is thus a measure of the fraction of O_2-evolving centers (*26*).

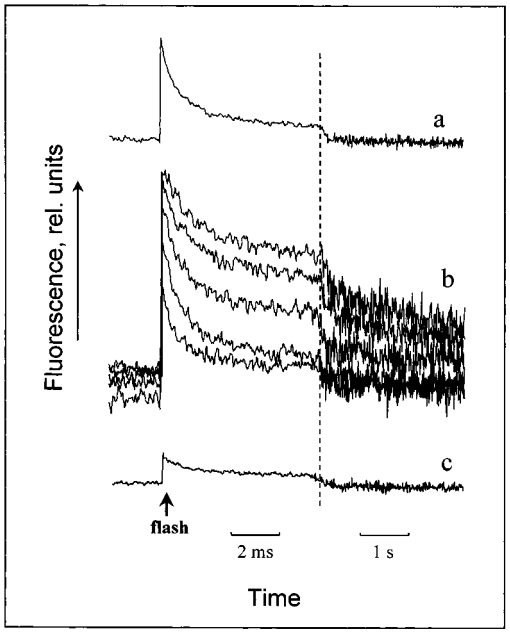

FIGURE 2: Flash-induced fluorescence decay kinetics from the thylakoid membrane (a), the B3 or grana (b), and the T3 or stroma lamellae (c) fractions. The curves represent averaging of 100 traces (10 independent samples × 10 flashes for each) in (a) and (c) while the curves in (b) represent averaging of 10 traces (in 10 independent samples) after 1 (bottom), 3, 5, 7, and 10 (top) flashes. The first 10 ms of the decay was measured at a modulation frequency of 100 kHz, beginning 2 ms before the actinic flash was given. The slower time scale was measured at a modulation frequency of 1.6 kHz. The actinic flashes were given at 10 s intervals.

Table 2: Flash-Induced Fluorescence Decay Half-Times and Amplitudes in the Different Subfractions of the Thylakoid Membranes

domain	flash no.	fast phase, ms[a]	middle phase, ms[a]	slow phase, ms[a]
thylakoids	all	0.39 (62%)	5.6 (38%)	nd[b]
grana	1	0.34 (57%)	5.9 (33%)	300 (10%)
core (BS)	5	0.47 (50%)	11.8 (36%)	510 (14%)
	10	0.71 (28%)	12.6 (28%)	930 (44%)
grana (B3)	1	0.29 (66%)	6.5 (34%)	nd
	5	0.42 (60%)	9.8 (40%)	nd
	10	0.52 (34%)	24.0 (29%)	775 (37%)
grana (B3)[c]	all	0.34 (67%)	6.4 (33%)	nd
margins	1	0.62 (53%)	46.0 (47%)	nd
	10	0.73 (57%)	113 (43%)	nd
stroma (T3)	all	0.33 (42%)	29.0 (58%)	nd
Y100	all	nd	29.0 (100%)	nd

[a] The numbers in parentheses represent the amplitude of the phase in percent of the total flash-induced fluorescence. [b] Not detected. [c] Measured in the presence of 2 mM plastocyanin or 2 mM ferricyanide.

were active in O_2 evolution. PSII in the margin fraction is a mixture of active and non-active centers, and this fraction has properties intermediate between those of the grana core and stroma lamellae fractions.

Flash-Induced Variable Fluorescence Decay Kinetics: Photosystem II Acceptor Side Reactions. Figures 2 and 3 show the flash-induced fluorescence curves from the different subfractions. In the intact thylakoid membranes, the fluorescence decay kinetics are not dependent on the number of flashes applied (Figure 2, a). The decay kinetics could be fitted with two kinetic phases: 0.39 and 5.6 ms (Table 2). These are comparable with literature values for the electron transfer from Q_A^- to Q_B (or Q_B^-) and to rebinding of

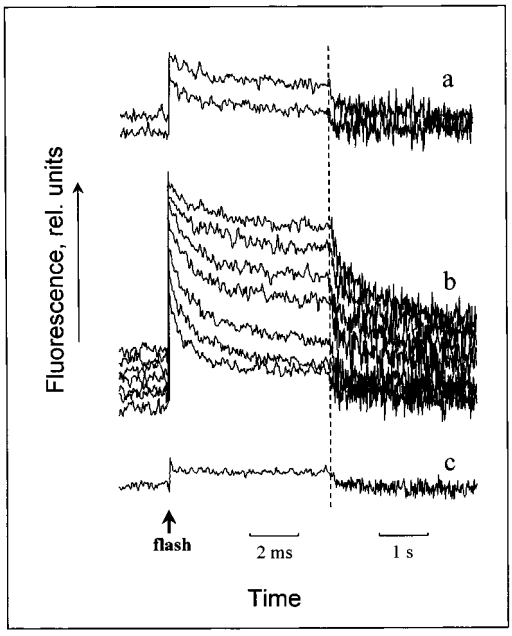

FIGURE 3: Flash-induced fluorescence kinetics from the margin domain (a), the BS or grana core (b), and Y100 (c) fractions of the thylakoid membranes. The measuring conditions were essentially as in Figure 2. The two curves in (a) represent fluorescence decay after the first (bottom) and tenth (top) flashes, and the curves in (b) represent fluorescence decay after 1 (bottom), 3, 5, 7, 8, 9, and 10 (top) flashes. The curve in (c) represents the decay after averaging of 100 traces.

plastoquinone to the Q_B site, respectively (see introduction; *16, 18, 29, 30* and references cited therein).

The flash-induced fluorescence kinetics from the intact grana (B3) fraction were different. In particular, the amplitude of the induced variable fluorescence was dependent on which flash number, in a train of flashes, was studied (Figure 2, b; Table 2). In the first flash, only about 50% of the maximally inducible F_V were induced. After 8−10 flashes, the variable fluorescence reached its maximal level and stayed the same if the flashing was continued. In addition, the level of F_0 fluorescence increased by 20% during the 10 flashes, indicating that, on each flash, a small fraction of Q_A stayed reduced until the next flash was applied. The decay kinetics changed depending on the number of flashes given. The fluorescence decay after the first flash to a dark-adapted B3 fraction showed two decay phases: 0.29 and 6.5 ms, quite similar to what was found in the intact thylakoids (Table 2). The fluorescence decay after 10 flashes showed 3 decay phases: 0.52, 24.0, and a new slow phase of 775 ms. The relative amplitude of the fast phase decreased from 66 to 34%, and the amplitude of the new, slow phase, was 37% after 10 flashes (Table 2). Again we assign the two fast phases to forward electron transfer from Q_A^-, and their decreasing importance with flash number (100% on the first flash and 63% on the tenth flash) suggests that the PQ pool is limited and that its reoxidation is not sufficiently fast in this preparation. This interpretation is further corroborated by the slowed binding of Q_B [from 6.5 ms on the first flash to 24 ms on the tenth flash (Table 2)]. The slow phase (775 ms) can be attributed to recombination of Q_A^- with the S states of the O_2-evolving complex in PSII (*17, 31*). It appears because recombination becomes important when the PQ pool is fully reduced after a few flashes. This was supported by

Table 3: Flash-Induced Fluorescence Decay Half-Times and Amplitudes for Different Fractions of the Thylakoid Membranes in the Presence of 40 μM DCMU

domain	fast phase, ms[a]	middle phase, s[a]	slow phase, s[a]	O_2-evolving centers (%)[b]
thylakoids	64 (27%)	1.36 (53%)	>10 (20%)	80
grana core (BS)	280 (30%)	1.23 (47%)	>10 (23%)	91
grana (B3)	170 (31%)	1.62 (53%)	>10 (16%)	84
margins	90 (32%)	1.29 (34%)	>10 (33%)	66
stroma (T3)	44 (31%)	3.50 (39%)	>10 (30%)	43
Y100	39 (100%)	nd[c]	nd	0

[a] The numbers in parentheses represent the amplitude of the phase in percent of the total flash-induced fluorescence. [b] Data from Table 1. [c] Not detected.

the observation that the flash number-dependent variable fluorescence rise and the very slow decay phase both disappeared when 2 mM plastocyanin or 2 mM ferricyanide was added as electron acceptor. In this case, we observed only two stable decay phases which were independent of the flash number (Table 2).

The situation is different in the T3 fraction from the stroma lamellae. Here, the variable fluorescence was totally independent of the number of flashes provided, similar to the situation in intact thylakoids (Figure 2, c), indicating that PSII could not fully reduce the PQ pool. We detected two kinetic phases: 0.33 and 29 ms (Table 2). The fast phase can again be attributed to the forward electron transfer from Q_A^- to Q_B (or Q_B^-). In contrast, the substantial 29 ms phase (58%) is quite slow for acceptor side reactions in a functional PSII complex, and we see several explanations to this phase. It might reflect unusually slow binding of plastoquinone to the Q_B site in PSII in the stroma lamellae due to an altered acceptor side. However, it is interesting to note that the amplitude of the 29 ms phase (58%) correlates closely to the fraction of non-O_2-evolving PSII centers in this subfraction (57%, Table 1). Thus, another explanation is that the 29 ms phase represents a recombination reaction with the donor side of PSII when the Mn cluster is impaired or absent (*20*, *22*, *32*, *33*). However, our measurements in the presence of DCMU (Table 3), which only detects Q_A^- recombination to the donor side, indicate that the $Q_A^-Y_Z^{ox}$ recombination is slower (44 ms) than the 29 ms phase (see below). Thus, it is likely that the 29 ms phase indeed represents very slow Q_B binding. The amplitude of this phase (58%, Table 2) coincides with the fraction of the centers that are incapable of oxidizing water (Table 1). This suggests that the slow binding of plastoquinone to the Q_B site occurs in centers deprived of the Mn cluster. Support for this conclusion comes from a recent study of PSII from dark-grown *C. reinhardtii* cells where the Mn cluster is lacking (*20*).

The flash-induced fluorescence kinetics in the margin vesicles were again dependent on the flash number (Figure 3, a; Table 2). The total fluorescence amplitude was increased by about 30% in 10 flashes, which is less important than the almost doubling that was observed in the B3 and BS fractions from the grana. Fitting of the decay curves shows the presence of two kinetic phases, 0.62 and 46 ms after the first flash and 0.73 and 113 ms after 10 flashes, which are both slow when compared to decay kinetics in the other membrane subfractions. Furthermore, the distribution of the fluorescence amplitudes between the two phases was hardly dependent on the flash number (Table 2). This is again very different from the situation in the grana preparations (B3 and BS) but quite similar to that from the stroma lamellae (T3) preparation. Taken together, these data indicate slow electron transfer between the quinones on the acceptor side of PSII in the margins. From these data alone, we cannot ascertain if the 46 ms phase only reflects forward electron transfer from Q_A^- to an empty Q_B site where Q_B first has to be rebound. However, in the presence of DCMU (see below, Table 3), the fastest recombination occurs with a half-time of 90 ms which most likely reflects recombination with Y_Z on the donor side of PSII. The 46 ms phase on the first flash is clearly faster. Thus, we conclude that this phase most likely reflects forward electron transfer, and consequently slow binding of Q_B in a fraction of the centers. After 10 flashes, the slow phase is longer (113 ms) and might reflect a mixture of slow forward electron transfer and $Q_A^-Y_Z^{ox}$ recombination. In this respect, it is important to note that a substantial fraction (34%) of the PSII centers in the margins were lacking O_2 evolution (Table 1).

The fluorescence decay kinetics in the highly purified grana core fraction (BS) were similar to those of the B3 fraction and were strongly flash number dependent (Figure 3, b). A total of 10−12 flashes were needed to reach a stable maximal fluorescence level. Three kinetically resolved phases were observed already after the first flash (Table 2). The forward electron transfer on the acceptor side of PSII was slower than in the B3 fraction: 0.34, 5.9, and 300 ms. After 10 flashes, we observed 0.71, 12.6, and 930 ms phases. The amplitudes of the decay phases changed with flash number with the same tendency as in the B3 fraction: the amplitude of the fast phase decreased from 57 to 28% while the amplitude of the slow phase increased from 10 to 44% in 10 flashes (Table 2). Thus, PSII in the core of the grana stacks (BS fraction) has a well-functioning acceptor side but a very limited PQ pool. It cannot be reoxidized because of lack of plastocyanin that slowly makes forward electron transport from Q_A^- almost impossible. This also explains the early appearance of the slow phase that reflects recombination with the Mn cluster. The presence of ferricyanide or plastocyanin eliminated both the flash-dependent buildup of fluorescence and the appearance of the slow recombination phase (not shown, but compare with intact grana in Table 2, plus ferricyanide or plastocyanin). This indicates that the PQ pool size and reoxidation of the plastoquinol limit acceptor side electron transport in PSII in the grana.

The flash-induced fluorescence in the Y100 fraction was very small (Figure 3, c; Table 1), and the fluorescence decay showed one 29 ms decay phase (Table 2). We were unable to resolve any decay in the microsecond time domain. Thus, there seems to be no electron transfer from Q_A^- to bound Q_B in PSII in this purified domain of the stroma region. The 29 ms decay half-time is quite similar to the fluorescence decay observed in the presence of DCMU (see below, Table 3). It can therefore probably be attributed to recombination between Q_A^- and Y_Z^{ox} due to the absence of the functional Mn cluster. The domination of this phase suggests that the Y100 fraction contains little or no PSII centers with a normal forward electron transfer. This is also consistent with the low F_V/F_0 and the lack of O_2 evolution (Table 1). Consequently, PSII centers in this purified part of the stroma lamellae are perturbed both on the acceptor side (very poor,

if at all existing, Q_B exchange) and on the donor side (no functional Mn cluster).

Flash-Induced Variable Fluorescence Decay Kinetics: Photosystem II Donor Side Reactions. In the presence of the inhibitor DCMU, recombination with components on the donor side will dominate the fluorescence decay that thus becomes a reporter on the integrity of the Mn cluster, Y_Z, and other electron donors in PSII (*15, 16, 20, 21, 33*−*36*). In intact thylakoids, the fluorescence decay showed 64 ms, 1.36 s, and >10 s decay half-times with 27, 53, and 20% of amplitudes, respectively. The fast phase was slower in the B3 fraction [170 ms (31%)] while the middle phase was approximately the same [1.62 s (53%)]. The amplitude of the very slow phase was also quite similar. In contrast, in the grana core (BS) fraction, the shortest recombination phases were slower and encompassed fewer centers as compared to the grana (B3) fraction: 280 ms (30%) and 1.23 s (47%). The situation was different in the stroma lamellae (T3) fraction—the fast phase was substantially shorter [44 ms (31%)] and the middle phase was slower and less important in amplitude (3.5 s, 39%). The decrease of the amplitude of the middle phase was accompanied with an increase of the very slow phase. In the Y100 domain, the flash-induced fluorescence decay showed only one 39 ms half-time decay phase (quite similar to the 29 ms decay half-time observed without DCMU, Table 2). It is noteworthy that the 39 ms decay phase in the Y100 fraction is similar to the 44 ms decay phase in the stroma lamellae fraction (Table 3).

In the margin fraction, the fluorescence decay half-times were similar to those in the intact thylakoid (Table 3), and the fast decay phase was clearly slower than in the fractions originating from the stroma lamellae.

In the presence of DCMU, the flash-induced fluorescence showed three resolved decay phases in all fractions except for the Y100 fraction (Table 3). The fast phase was in the 60−300 ms time scale (in stroma lamellae and Y100, ∼40 ms) while the middle phase occurred in the 1−3 s time scale. In addition, part of the decay was very slow (more than 10 s, Table 3). The middle phase can be assigned with certainty to recombination between Q_A^- and the S_2-state of the O_2-evolving centers. The origin of the fast phase is less clear. In the stroma lamellae, the middle decay phase (39%, Table 3) closely coincides with the fraction of O_2-evolving centers (43%, Table 1), and here we assign the fast decay phase (44 ms) to recombination with donor side components without a functional Mn cluster. This decay time is very similar to the observed decay in the Y100 fraction (39 ms, Table 3) which encompassed all recombining centers in accordance with the complete lack of O_2-evolving centers in this fraction.

Very slow fluorescence decay phases can have a different origin, and a very slow phase has been observed in the PSII lacking the Mn cluster (*22, 32, 33, 37, 38*). Two principally different explanations have been offered—the electron remains on Q_A due to the absence of a recombination partner or due to the action of an electron donor which reduces the donor side of PSII but which does not participate in recombination reactions. It is often difficult to differentiate between these possibilities, but it is likely the slow phase represents different reactions in our different fractions (see Discussion).

To conclude, we found that the donor side components involved in recombination reactions in PSII from the different domains of the thylakoid membranes were quite different, similarly as was the case with the acceptor side reactions.

DISCUSSION

One approach to study the structure and integrated function of a system as complex as the thylakoid membrane is to fragment it, separate the fragments, and study their biochemical and biophysical properties. We have chosen to apply mechanical fragmentation and sonication in combination with aqueous polymer two-phase separation. This work therefore complements other studies where detergents are used (for example, BBY-type preparations).

We will discuss our results at two levels. First the properties of PSII in each subfraction will be analyzed; then we will focus on the dynamic nature of the thylakoid membrane and try to address why PSII is different in different membrane compartments.

Leaves contain PSII in many functional states. In particular, the photoinhibition−repair cycle (*40* and references cited therein) gives rise to a heterogeneous pool of PSII centers that varies over time. This mix can be assessed by different techniques, and the studies of flash-dependent variable fluorescence applied here focus our investigations to centers that either are fully functional or have entered in the repair part of the cycle. Centers that have suffered photoinhibition are normally inactive in variable fluorescence and will consequently not show up in our present results. This is important to remember when analyzing the complex data set.

Both the donor and the acceptor side of PSII differ in the fractions studied. The most active PSII centers are found in the appressed region of the grana (B3 and BS fractions), in agreement with the current consensus of the thylakoid membrane organization (*25, 39, 41*−*43*). The electron transport between Q_A and Q_B is efficient (Table 2), but after very few flashes, the PQ pool is completely reduced (Figures 2, b, and 3, b), resulting in much slower electron transport from Q_A to Q_B (Q_B^-) and the appearance of slow recombination reactions to the donor side (Table 2).

PSII from the granal part of the membrane (B3 and BS) was almost completely (85−90%) competent in O_2 evolution (Table 1). Consequently, the fluorescence decay kinetics in the presence of DCMU (Table 3) should be dominated by back-reactions between Q_A^- and the Mn cluster. We observe three decay phases in the variable fluorescence. The middle second phase, which encompasses a large fraction of the centers, reflects recombination between Q_A^- and the S_2 state. The very slow phase (>10 s, ∼20%) is of less defined origin. However, in dark-adapted material, about 20−25% of PSII is found in the S_0 state [or has Y_D reduced (*44*)] while the remaining 75−80% is in the S_1 state (*45*). Upon a flash, these centers turn over to form $S_1Q_A^-$. The S_1 state does not recombine with Q_A^-, and it is probable that the slow phase represents intact O_2-evolving centers in the grana-originating preparations.

The fast fluorescence decay in the grana preparations (Table 3, 30%, 200−300 ms) is unusually fast to represent $Q_A^-S_2$ recombination. However, it is also unusually slow to represent Q_A^- recombination with $P680^+$ or Y_Z^{ox} in centers

lacking the Mn cluster. It is also interesting that the decay half-time is longer (280 ms, Table 3) in the grana core, BS, than in the preparation of entire grana, B3 (170 ms, Table 3).

It is noteworthy that similar recombination half-times (100−300 ms time range) have been observed early during photoactivation of PSII in *C. reinhardtii* (*20*). It was also found that these recombination half-times increased in parallel with photoactivation due to the stepwise buildup of the Mn cluster during the photoactivation (*20*). We interpret our present results similarly. Our analysis does not report on photoinhibited centers (see above). Therefore, we propose that the centers that show Q_A^- recombination in the 200−300 ms time scale have partial Mn clusters and are in the process of becoming fully functional.

The question is then: What are the properties of the Mn cluster in this population of PSII in the grana (30% of PSII)? A control experiment where the electron donor DPC was used revealed that the fluorescence decay kinetics in the presence of DCMU did not change (not shown). This suggests that all PSII centers in the grana region showing variable fluorescence have Mn bound to its site; i.e., they are either fully functional or partially photoactivated. The partially photoactivated centers must be quite stable to allow their detection after the preparation procedure. It is even possible that these not completely photoactivated O_2-evolving centers will become completely photoactivated during the first turnovers in the steady-state O_2 evolution assay. The latter suggestion is supported by the observation that the 280 ms recombination phase (Table 3) becomes less important after more flashes (not shown). Thus, in the granal preparations, the absolutely dominating fraction of the grana centers contains a Mn cluster which is fully functional or partially assembled.

In the margin fraction, the acceptor side of PSII is functional, but the electron transport is slow (Table 2). The flash-dependent changes in the fluorescence behavior are small (Figure 3, a), which indicates that there are enough electron transfer carriers from PSII (PQ pool, cytochrome *b/f* complex, plastocyanin, and PSI) in this part of the thylakoid membrane. The margin fraction behaves similarly to the entire thylakoid but with a larger population of non-O_2-evolving centers [Tables 1 and 3 (*25*, *46*)]. In the margins, about 35% of the centers are not active in O_2 evolution. It is possible that these centers, in part, have slow electron transfer from Q_A^-, similar to what has been found in PSII from dark-grown cells without a functional Mn cluster (*20*) and in PSII-enriched membranes depleted of the Mn cluster (*47*, *48*). The inactive centers (∼35%) give rise to the 90 ms fluorescence decay in the presence of DCMU (Table 3) which we in this case assign to $Q_A^-Y_Z^{ox}$ recombination. The middle and slow decay phases are assigned to PSII centers with a functional Mn cluster which are active in O_2 evolution [66% of the PSII centers are O_2-evolving (Table 1) while the total amplitude of the middle and slow fluorescence decay phases (Table 3) is 67%]. Our results correlate well with thermoluminescence measurements in the presence of DCMU in the margin fraction (*49*). These show that the Q-band (which is ascribed to recombination between Q_A^- and S_2) accounted for 60% of the thermoluminescence while the C-band (which is ascribed to $Q_A^-Y_D^{ox}$ recombination) amounted to 40%. This also provides further strength to our estimation of the O_2-evolving centers and to our assignment of the fluorescence decay phases in the margin fraction.

Consequently, we conclude that the margins contain a mixed population of PSII centers. A large fraction is not functional in O_2 evolution and is only able to slow forward electron transfer from Q_A^-. In both respects, they are very similar to PSII from dark-grown *C. reinhardtii* (*20*). A tempting hypothesis is therefore that the nonactive PSII centers in the margins are in the process of becoming photoactivated. In contrast to the non-O_2-evolving centers in the grana, the nonphotoactivated PSII centers in the margins have no or very little bound Mn. Most likely they represent assembled PSII centers with active electron transport on the acceptor side but before the Mn cluster has begun to become activated. Our suggestion is that the margin region, situated between the granal and stromal parts of membrane, is very important for recovery and photoactivation of PSII. Probably, the first stable intermediates during photoactivation start to accumulate in this membrane region.

In the nonapressed membranes, there seems to be a gradual increase in the functional abilities of the PSII centers. In the most purified fraction, Y100, there are no O_2-evolving centers. Furthermore, there is hardly any forward electron transfer from Q_A^-. The fluorescence decay half-times in the Y100 fraction are similar in the presence and absence of DCMU (39 and 29 ms, respectively). In the presence of DCMU, this most probably represents recombination between Q_A^- and Y_Z^{ox} in centers totally lacking the Mn cluster. Thus, the small amount of PSII in the Y100 fraction is unable to oxidize water, totally lacking Mn, and very inefficient (or unable) in forward electron transfer. This is in agreement with previous reports that the non-Q_B-reducing centers are located in the stroma lamellae (*27*, *49*). The thermoluminescence study (*49*) reported only a very small amount of the Q-band (O_2-evolving PSII centers) and a dominating C-band (representing centers without the Mn cluster) in the stroma lamellae. Our interpretation is also coherent with the observation that centers without a Mn cluster have an increased potential of Q_A^- (*47*) that slows down forward electron transfer from Q_A.

In the preparation of the entire stroma lamellae (the T3 fraction, Figure 1), PSII seemingly functions better than in the Y100 fraction although less well than in the margin fraction, and almost half of the centers have Q_A^- well connected to Q_B (330 μs, 42%; Table 2). This correlates very well with the 43% of centers that were active in O_2 evolution and also with the centers (39%) that show quite normal $Q_A^-S_2$ recombination (middle kinetic phase, Table 3). Consequently, about 40% of the centers are fully assembled functional PSII centers. The remaining centers are unable to show O_2 evolution. About 30% of the centers seem to be devoid of any functional Mn as judged from the very fast (44 ms, Table 3) recombination in the presence of DCMU. These centers probably are very slow in forward electron transfer (29 ms, Table 2) and are thus almost identical to the centers which dominated in the Y100 fraction (which is included in the T3 preparation, see Figure 1). Consequently, we can conclude that PSII in the Y100 fraction does not constitute more than 30% of the PSII centers in the stroma lamellae. Thus, the T3 fraction contains about 40% fully active PSII centers and 30% centers that probably are very young and not yet photoactivated to any extent. What is then

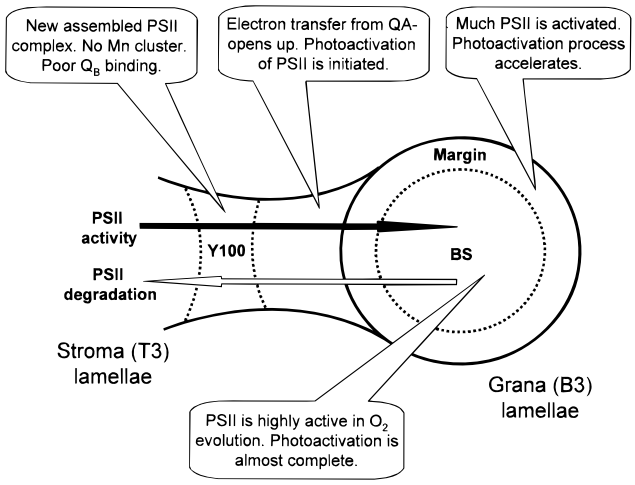

FIGURE 4: Overview of the PSII repair and photoactivation processes in the different domains of the thylakoid membrane.

the condition of the remaining PSII (30%) in this intermediate, complex membrane subfraction? The PSII centers are not efficient in forward electron transfer (Table 2, 29 ms), which suggests that the Q_B binding site is altered (*20*) and the redox potential of Q_A^- is probably upshifted as in Mn lacking PSII (*47*). The centers do not evolve O_2, but, in contrast to centers entirely lacking the Mn cluster, the recombination between Q_A^- and the donor side is very slow (>10 s in 30% of the centers, Table 3). Similarly, very slow recombination phases have been observed before and might represent different situations. These include functional centers in S_0 before the flash (see above), electron donation from an exogenous donor like DPC or Mn^{2+}, or more likely in the T3 fraction, electron donation from an endogenous donor in PSII like Y_D or cytochrome b_{559}, which both are poor recombination partners to Q_A^-. In this respect, it is very interesting that both Y_D and cytochrome b_{559} recently were found to be crucial early electron donors to PSII during photoactivation (*50*). They execute their function at the moment when the forward electron transfer is becoming functional but when the Mn cluster still is not functionally bound. We hypothesize that the T3 fraction contains a large fraction of PSII in this fragile state.

CONCLUSIONS

A dynamic and complex picture of the status of PSII during its lifecycle emerges from this study. Some parts of this dynamic process have been proposed earlier (see, for example, *51, 52*), but our main conclusion relates to the centers that are becoming active in the assembly and repair process of PSII. Figure 4 provides an overview of the PSII activity gradient in the thylakoid membrane proposed here.

PSII starts to assemble in the part of the stroma lamellae we have denoted Y100. Here, PSII has no Mn cluster and is unable to forward electron transfer from Q_A^-. PSII then migrates toward the granal fraction of the membrane, and we interpret our results to indicate a stepwise activation of PSII. When we prepare the entire stroma lamellae, this contains a fraction of fully activated PSII and an intermediate fraction of PSII (~30%). The latter centers are becoming functional on the acceptor side but lack any bound Mn cluster (Figure 4). It is likely that these centers are located to the part of the stroma lamellae found closest to the grana. In the margins, we find no trace of PSII centers lacking Mn. Instead two-thirds of the centers are fully functional while the remaining centers probably contain partially photoactivated Mn clusters. In the grana stacks, the centers are even more active and only a small fraction of the PSII is still inactive in O_2 evolution.

Consequently our study complements work directed on photoinhibitory reactions and provides a coherent, full picture of the photoinhibition repair cycle. Irreversibly inhibited centers migrate from the grana to the stroma lamellae. Here, the D1 protein is excised and degraded (not studied here; but see *40* and references cited therein). PSII is then partially assembled to a small center lacking antennae and the Mn cluster. We suggest that this occurs in the Y100 fraction. From this part of the membrane, PSII then migrates back to the margin. This occurs concomitantly with activation of the acceptor side and photoactivation of the Mn cluster. The final steps of the PSII recovery take place in the grana, and fully active PSII centers then continue their life cycle.

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