2. MATERIALS AND METHODS

2.1 Buffers

All reagents used in this work were of analytical or high purity grade (99.97% - 99.999%). The reagents were purchased from Sigma, Aldrich, Fluka, Pharmacia, and other companies.

2.2 Preparative procedures

Samples prepared from *Spinacia oleracea* (spinach) were used throughout this work. Fresh spinach was bought from the market and sample preparations were done at the same day. All isolation procedures were done on ice and under dim green light or in darkness. The temperature in all centrifugations was 4°C, unless otherwise stated.

2.2.1 Isolation of oxygen-evolving PSII-enriched membranes.

The isolation of PSII membranes from spinach was carried out according to the following protocol:

Cell Disruption: 2.5 kg of fresh spinach were washed in ice-water, intact spreads of green leaves were separated from the stem. The leave material was mixed with 1 l of Buffer A (ascorbat and fresh BSA were added) in a blender (GT 800). The suspension was homogenized for 20-30 sec. at the highest mixing speed. The homogenate was filtrated and strongly extruded through three layers of cheesecloth. The filtrate was passed through three layers of cheesecloth and two layers of thin filter and centrifuged for 10 minutes at 6200 x g (SLA3000).

*Chloroplast disruption and thylakoid extraction:*The supernatant was discarded and only the dark green part of the pellet was resuspended using a brush and homogenized an equal volume of Buffer B, reaching a total volume of 300 ml. The suspension was centrifuged for two minutes at 1100 x g (SS-34). The pellet was discarded and the supernatant was first centrifuged for two minutes at 4400 x g (SS-34) and finally for 5 minutes at 50000 x g (SS-34). The pellet was homogenized with about 40 ml of Buffer C.

Grana purification: The total volume was measured and the chlorophyll concentration was determined. Both Buffer C and Triton X-100 (stock solution) were added to reach a final chlorophyll concentration of 2 mg Chl/ml and a ratio Triton X-100 to Chlorophyll of 25:1 (w/w). The addition of the Triton X-100 was carried out slowly with a pipette (dropwise with gentle stirring for 1 minute on ice) to solubilize the stromal membranes. The suspension

was centrifuged for two minutes at 1100 x g (SS-34). The pellet was discarded and the supernatant was centrifuged for 12 minutes at 50000 x g (SS-34).

Purification and storing of PSII membrane fragments: The supernatant was discarded and the starch-free part of the pellet was homogenized with a brush in Buffer D. The suspension was again centrifuged for 12 minutes at 50000 x g (SS-34). This step was repeated until the sample was free of starch (at least 2 times). Finally, the pellets were suspended in 20-50 ml Buffer D adjusting the chlorophyll concentration to 2 -3 mg chl/ml. Chlorophyll concentration was determined and the membrane particles were stored at - 80°C.

The typical yield of the PSII preparation was about 100 mg chlorophyll with an oxygen activity between 1200 -1400 µmol O_2 (mg Chl x h)$^{-1}$ and a Chl *a/b* ratio of 2.2 - 2.4.

2.2.2 Betaine removal from PSII membrane particles

PSII-enriched membrane particles were thawed on ice in the dark for 2 hour, and then the PSII particles were resuspended and diluted in Buffer E (total volume about 300 ml). The suspension was centrifuged for 20 min at 30000 x g, using eight centrifuge tubes in a Sorval SS-34 rotor at 4°C. The supernatant was discarded and the pellet carefully resuspended in Buffer E and again centrifuged. This step was repeated three times.

2.2.3 Depletion of extrinsic polypeptides (18 and 23 kDa)

Betaine-free PSII membrane particles were resuspended at 1 mg Chl/ml in Buffer F and incubated on ice (4°C) in the dark for 30 min, gently stirred. The solution was centrifuged at 50000 x g for 20 min. The supernatant was collected and stored (at - 80°C), it contained the depleted extrinsic polypeptides (18 and 23 kDa) (Miyao and Murata, 1983). The pellet was resuspended in Buffer G.

2.2.4 Extraction of Manganese from PSII

Two experimental procedure were used for the removal of Mn from PSII preparations: **a)** Complete Mn extraction using a strongly alkaline CHES Buffer (pH 9.4)/$MgCl_2$ wash and **b)** Partial Mn extraction using a mild hydroxylamine (pH 6.8) wash.

a) Complete extraction of Mn (CHES wash)

Betaine-free PSII membranes were resuspended in 16 ml of Buffer H (0.25 mg Chl/ml) and gently stirred at room temperature under room light for 10 min. The sample was diluted 1 : 1 with 50 mM MES buffer, pH 6.5, to stop the extraction and then was centrifuged at 30000 x

g for 20 min. The pellet was washed once in Buffer I to which 1 mM EDTA was added. Subsequently, the pellet was washed in Buffer I and resuspended in the same buffer to about 2-4 mg Chl/ml for analysis or storage in nitrogen liquid.

b) Partial extraction of Mn (NH_2OH wash)

Betaine-free PSII membranes were resuspended in 16 ml of Buffer J (0.25 mg Chl/ml) and were gently stirred on ice in the dark for 10 min. Subsequently, after centrifugation the pellet was washed in Buffer I and resuspended to reach about 2-4 mg Chl/ml.

2.2.5 Extraction of the functional calcium from PSII (citrate wash)

Ca-depletion treatments were performed according to the low-pH/citrate procedure of (Ono and Inoue, 1988). PSII membrane particles in Buffer K were pelleted by centrifugation (15 min at 40000 x g) and then resuspended to approximately 6 mg of Chl/ mL in Buffer L. The particles were then diluted to 2 mg of Chl/mL in Buffer M. After of 5 min incubation at 0 °C, the preparation was brought to pH 6.5 with Buffer N and pelleted by centrifugation (15 min at 40000 x g). The membranes were then resuspended in Buffer N and pelleted again. The final step before sample preparation was resuspension in Buffer O. The resuspensions were performed in the dark and were homogenized slowly to ensure uniform suspension.

2.2.6 Disassembly of PSII by a temperature jump (heat treatment)

a) Preparation of multilayer samples

1) Pre-purification: Frozen suspensions of PSII membrane particles (3-4 mg of Chl/ml) were slowly thawed on ice in the dark for 1 -2 hours and resuspended in Buffer D. The suspension was centrifuged for 12 minutes at 50000 x g (SS-34).

2) Concentration adjustment: The pellets were resuspended in Buffer D plus 10 % glycerol, the total volume was measured, and the chlorophyll concentration was determined. PPBQ (stock solution) was added up to the desired concentration. Buffer D and glycerol were added to reach a final concentration of 1 mg Chl/ml. PPBQ, an electron acceptor, was previously recrystallized in fresh ethanol (see Appendix).

3) Filling of the frames: Three frames (acrylic-glass) were placed adequately in the hemispheric cap (PVC component) (see Figure 2.1 (A)) and coupled to the cylinder

(PVC with rectangular hole) (Figure 2.2 (B)) on the inside of the centrifuge tube (Figure 2.2 (C)). Each frame was filled with 1 mg of Chl and subsequently centrifuged for 15 minutes at 71000 x g (SW-28) at 4 °C. The supernatant was discarded and the cylinder was removed. 120 ml of Buffer D plus 10% (v/v) glycerol were added together with PPBQ (stock solution) until the desired concentration was reached. Centrifugation for 1 hour and 45 minutes at 112000 x g (SW-28) was performed. The supernatant was discarded and the hemispheric cap was carefully removed.

4) Drying and storage: The frames were placed in a desiccator. Silica gel was placed at the bottom of the desiccator, which was connected to a membrane pump (MZ 2C, vacuubrand) to lower the pressure to 250 mbar. The frames were dried in the dark in a cold room at 4°C for 1.5 hours and stored in liquid nitrogen thereafter.

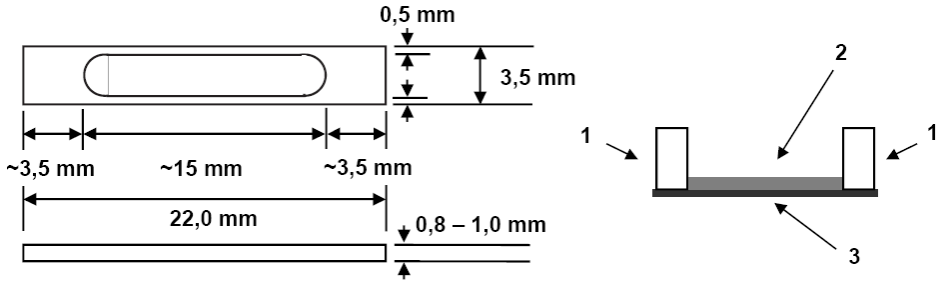

Figure 2.1 Schematic diagram of acrylic-glass frames for multilayer. 1: Sample carrier; 2: Cross section for PSII membrane particles: 3: Kapton foil. Adapted with permission from (Müller, 2006b).

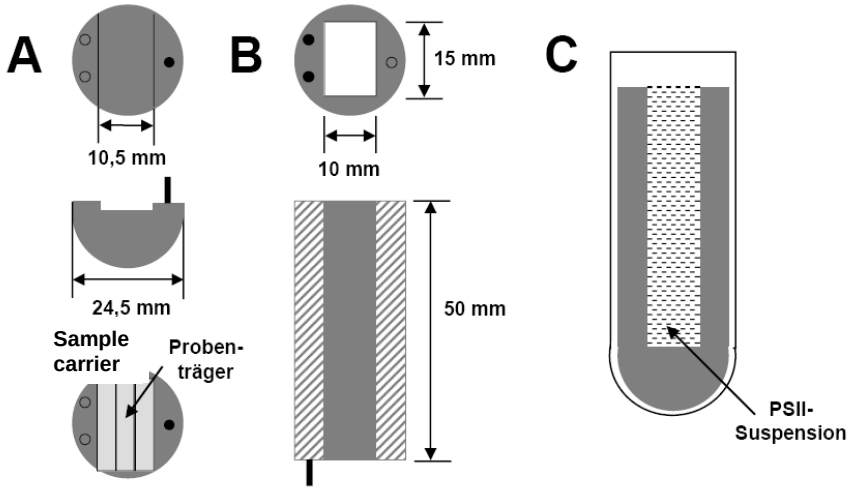

Figure 2.2 Schematic diagram of the PVC tube for ultracentrifugation of the multilayer samples. (A) Hemispheric part, which contains three acrylic-glass frames (B) cylinder with reactangular hole section, which is fixed to the hemispheric component (C) view inside of the centrifuge tubes. Adapted with permission from (Müller, 2006b).

2.2.7 Heat treatment of multilayer samples

Frozen PSII multilayer samples (acrylic-glass frames) were thawed for 1 min at room temperature. Samples were enclosed in Eppendorf cups and exposed to 47°C by immersion in a digitally controlled thermostated water bath for 0–180 min. Immediately after the heat treatment, the samples were rapidly frozen (within less then 5 s) in liquid nitrogen (freeze-quench procedure for XAS and EPR samples). For the activity assay, the samples were rapidly put on ice, resuspended (sample temperature always close to 0°C), and then immediately used for the polarographic measurements. This procedure of heating was the result of extensive variation of heating temperature and time followed by characterization of the heated samples by chlorophyll fluorescence measurements (see (Pospisil and Dau, 2000)). At 47°C and for the indicated heating times, the inhibition of oxygen evolution in betaine-free samples was slow enough to be resolved.

2.2.8 Heat treatment of PSII membranes in solution

PSII membranes particles previously prepared with betaine as stabilizer (Papageorgiou et al., 1991; Allakhverdiev et al., 1996; Schiller and Dau, 2000) were thawed on ice for 2 hours and washed three times in Buffer E. Then, the obtained pellet was resuspended in Buffer E at (1 mg chl/ml), 700 µl were enclosed an Eppendorf cup and immersed in a digitally controlled water bath at 47 °C for 0 to 180 min. Heating was stopped by rapid cooling of samples on ice and PSII membranes particles were collected by centrifugation (50000 x g, 15 min, 4 °C). For the activity assay, the samples were resuspended in Buffer D, and then immediately used for the polarographic measurements.

2.2.9 Photoactivation

a) *PSII membrane particle preparations and Mn depletion*: PSII-enriched membrane particles were prepared routinely as described previously (2.2.1) and subsequently betaine was removed. The Mn depletion was carried out as described in 2.2.4. Unless otherwise stated, all subsequent operations were done at 4°C in the dark.

b) *Incubation with "reactivating" agents*: The samples (1 ml) containing 1µM PSII (according to an estimated content of ~200 Chlorophylls per reaction center) were incubated in Buffer P in the presence of 10 µM DCIP (external electron acceptor) and 5 mM $CaCl_2$. $MnCl_2$ was added from stock solution at concentrations indicated in the following sections. During the

preillumination, the suspensions were loaded into the photoactivation cell and immediately stirred in the dark at room temperature for 5 min (as indicated in Figure 2.3).

c) *Illumination conditions*: Weak continuous white light from a lamp was focused onto the cell. A standard photon flux of 30 μE $m^{-2}s^{-1}$ was measured at the sample surface and permanently controlled by a photometer (LI-185B LI-COR, Inc.), which was placed next to the cell. This intensity was sufficient to just saturate the photoactivation process.

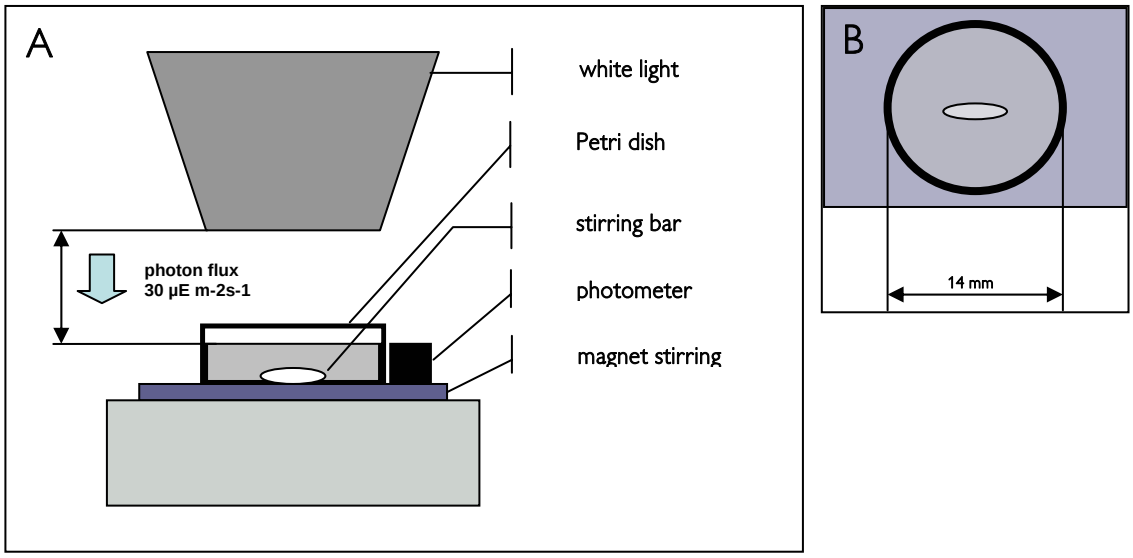

Figure 2.3 Schematic diagram of the photoactivation device. (A) General view of each component involved in photoactivation process. (B) Details of Petri dish with respective dimensions.

d) *Assay of O_2 evolution capacity*: Measurements of O_2 evolution were carried out polarographically essentially as described in 2.3.4. In experiments on the kinetics of photoactivation, 30-μl aliquots of PSII membrane suspensions (250 μl Chl/ml) were removed every two minutes from the photoactivation cell, added directly to the polarograph vessel, and immediately assayed.

2.3 Analytical Methods

2.3.1 Chlorophyll determination

Determination of the chlorophyll content in PSII membrane particles was done according to (Porra, 1990) with small modifications.

The chlorophyll from spinach preparations was extracted in 80% acetone /20% water. A centrifugation step in a Minifuge (Rotofix 32, Hettich) at 1600 x g / for 5 min was performed to remove the protein and lipids. Absorption spectra of the supernatant were measured against acetone solution at 646.6 nm, 663.6 nm and 750 nm using a UV-Vis-spectrophotometer (Cary 50 Conc, Varian).

Absorption (A) values were analysed using the following equations:

Total [Chl] : $[17.75(A_{646.6} - A_{750}) + 7.34(A_{663.6} - A_{750})]\ k$

[Chl a] : $[12.25(A_{663.6} - A_{750}) - 2.55(A_{646.6} - A_{750})]\ k$

[Chl b] : $[20.31(A_{646.6} - A_{750}) - 4.91(A_{663.6} - A_{750})]\ k$

k is a dilution factor (k=µl Buffer acetone/µl sample). Additionally, the ratio of [Chl a / Chl b] was calculated.

2.3.2 Sodium **d**odecyl **s**ulphate **p**olyacrylamid **g**el **e**lectrophoresis

a) *Gel preparation*: The stock solutions for SDS-PAGE were prepared as shown in Table 1 (Appendix) and stored at room temperature. 10% (w/v) APS solution was stored at - 20°C until use. A conventional Mini Protean II system (Bio-Rad, 10.5x10x0.75 cm gels) was used. The gel solutions are presented in Appendix.

b) *Sample preparation*: PSII membrane particles were prepared and the membrane lipids and chlorophylls, which disturb the resolution of smaller polypeptides, were removed by the following procedure. Samples (30 µL) were mixed with 100 µL of methanol by vortexing, then 900 µL of ethyl ether was added. After vortexing the mixture was centrifuged at 10 000 x g for 10 min. After removal of the supernatant, denaturing solution (described below) was added immediately and the polypeptides were dissolved using a vortex and/or a sonicator. When the concentration of polypeptides was low so as to exceed 30 µL/ml of lipid extraction mixture, the polypeptides were precipitated by 8% of trichloroacetic acid in order to decrease the

sample volume before the addition of methanol. The denaturing solution sample buffer was added to visualize the migration front in electrophoresis. PSII membrane particles containing 10 µg of protein (determined by the method of Lowry (1951)) were applied on the gels. Chlorophyll a concentration (Chl a) was determined by the method described above (Porra, 1990) (Figure 2.4).

c) *Electrophoresis conditions*: The Running Buffer (Appendix A) was the same as in Laemmli's system (Laemmli, 1970). A Mini Protean 3 Cell (10.5 x 10 x 0.75 cm^3) system with the samples was connected to a Pharmacia EPS 3500 power supply at 80 V for 4 h usually applied in case of slab gels containing 18% acrylamide and 6 M urea. The gel was stained by 0.15% Coomassie Brilliant Blue R-250 (Fluka, Germany) for 50 min in a solution of 50% methanol and 10% acetic acid, which was previously warmed to about 50°C to accelerate staining, and destained by the solution of 25% methanol and 7.5% acetic acid, which was also previously warmed to about 50°C to accelerate destaining.

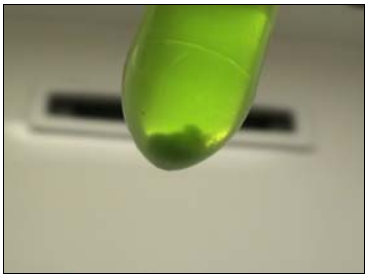

Figure 2.4 Eppendorf cup after delipidation process. The pellet contains the proteins which were precipitated and the supernatant contains chlorophylls and lipids that lower the resolution in the typical SDS-Page method.

2.3.3 Densitometry analysis

Densitometry analysis by a gel-scan technique developed by Dr. Peter Liebisch in our laboratory was used to quantify the protein bands in electrophoresis. Gels were digitized (TIFF image, 1200 dpi resolution) with a commercial scanner (HP scanjet 7400C). From these images, the integrated optical density assigned to each band was determined using a MATLAB program.

2.3.4 Oxygen evolution measurements

Oxygen evolution was assayed using a Clark-type electrode (5331 oxygen probe, YSI; High Sense Membrane) under saturating continuous white-light illumination from a tungsten lamp passed through a heat filter. The electrode was calibrated using sodium dithionite ($Na_2S_2O_4$). The measurements were conducted at 28°C. The reagents were added in the following order:

(1) 1.5 ml Buffer OEM (pH 6.3),

(2) 1 mM potassium ferricyanide (K_3 [Fe (CN)$_6$]),

(3) PSII membrane fragments at chlorophyll concentration of 5 mg/ml and

(4) 250 µM 2,6-dichloro-p-benzoquinone (2,6-DCBQ).

The oxygen measurements were recorded on a plotter (Servogor 330, BBC Goerz).

The oxygen evolution activity was calculated in [µmol O_2 x (mg Chl)$^{-1}$ x h^{-1}] using the following equation:

$$Oxygen \cdot activity = \frac{kxS(O_2) \times V \times 60}{Y \times C_{Chl} \times F_V}$$

where k is the slope of the measured trace; S (O_2) is the solubility of oxygen in air satured water at 28°C and standard atmospheric pressure (245 µmol l^{-1}) (Estabrook, 1967); C_{Chl} is the chlorophyll concentration (µg/ml); V is the speed of the chart recorder (cm/min^{-1}); Y is the amplitude of the calibration curve with sodium dithionite ($Na_2S_2O_4$); F_V is an amplification factor.

2.3.5 Western Blotting

After separation using polyacrylamide gel electrophoresis, the selected proteins were electrophoretically transferred from the gel to a membrane using the following procedure.

a) *Preparation and membrane transfer*: In order to make the proteins accessible to antibody detection, they were transferred from the gel onto a membrane made of PVDF. All procedures were carried out using protecting gloves.

Pieces of PVDF membrane (Millipore, Immobion) and blotting papers were sliced to the size of the gel to be blotted, in order to minimize the surface area exposed to the electrode: this reduces the amount of current that needs to be passed in order to achieve effective transfer.

Both membrane and blotting papers were wetted in Transfer Buffer at room temperature for 30 min.

The Transfer Buffer was removed adding 1 x Blotting Buffer until ready to use.

The assembly from top (cathode, (-)) to bottom (anode (+)) was performed in the following order (see Figure 2.5):

- Blotting paper
- SDS-PAGE gel
- PVDF membrane
- Blotting paper

A small roller was used in order to remove air between gel and membrane, and between membrane and blotting paper.

The transfer was performed in a Trans-Blot Semi-Dry (BioRad) at 500 mA (4°C) for 1 hr.

The membrane was resuspended in Blocking Buffer for 1 hour. This procedure allows saturation of all non-specific protein binding sites on the blots.

The membrane was washed in Wash Buffer twice for 15 minute with gentle agitation (Multi 3D-Shaker, Kisker).

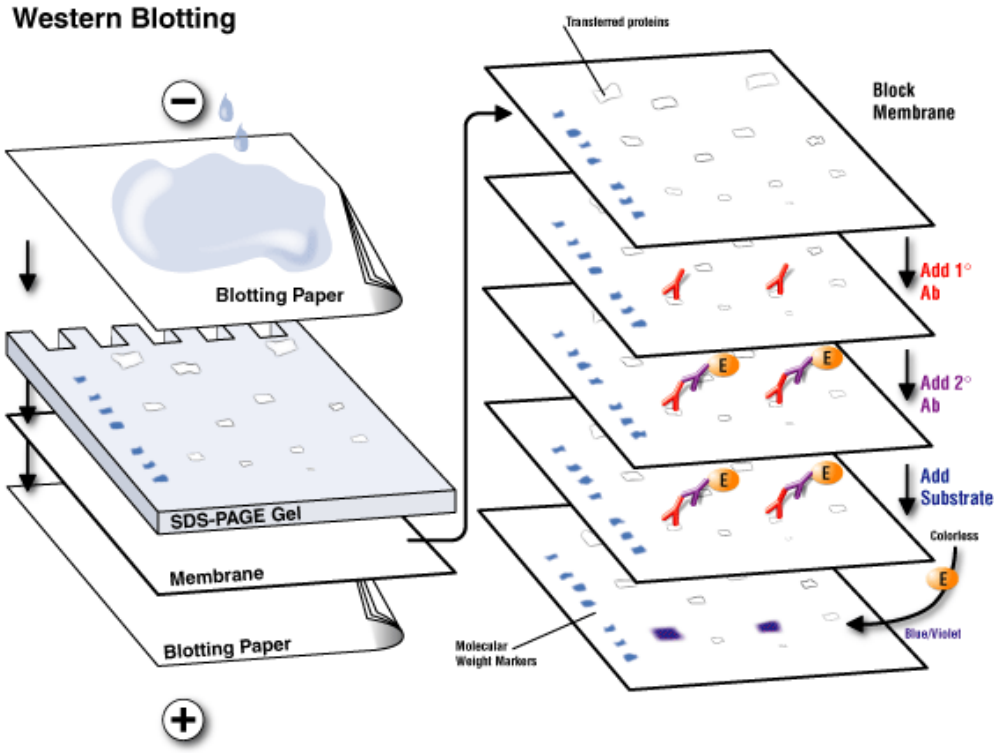

Figure 2.5 Diagram of assembly of the layers for the transfer membrane.

b) *Antibodies and detection*: 1) The membrane was incubated with primary antibody (10 ml, diluted 1:5000[a]) in Blocking Buffer for 1 h at room temperature.; 2) The membrane was washed in Wash Buffer three times for 10 min; 3) The membrane was incubated with the secondary antibody (10 ml, diluted 1:2500[a]) in Blocking Buffer at room temperature for 1 h. 4) The membrane was washed in Wash Buffer three times for 10 min; 5) The membrane was washed in Alkaline Buffer under gentle agitation for 2 min 6) The membrane was gently incubated in DETEC BUFFER until the bands were intense enough. 7) Finally, the membrane was washed in Milli-Q water and subsequently dried.

2.3.6 Graphite furnace atomic absorption spectroscopy (GF-AAS)

Graphite furnace atomic absorption spectroscopy was employed for quantification of manganese. In the PSII samples using an AAS 800 spectrometer (Perkin Elmer, Germany). Mn standard solution (Fluka, Germany) and samples were diluted with 0.2% (v/v) HNO_3. Measurements were carried out in the presence of a matrix modifier (3 µg Mg $(NO_3)_2$/20µl diluted sample solution) at 279.5 nm with a slit width of 0.2 nm, using the temperature program recommended by the manufacturer. Data handling and evaluation was done with AAwinlab (Perkin Elmer, Germany). The used protocol has been described in (Müller, 2006a). All measurements were performed in the laboratory of Dr. Klaus Irrgang (TU-Berlin).

2.3.7 Delayed fluorescence measurements

Previously thawed the samples were resuspended at a Chl concentration of 10 µg/mL. Before the measurements, the electron acceptor DCBQ was added to a concentration of 20 µM.

In delayed fluorescence (DF) experiments (Grabolle, 2005b), the dark-adapted samples were excited by saturating Laser flashes of 2 mJ/ cm^2 (Continuum Minilite II; λ =532 nm; fwhm of 5 ns; time between flashes of 0.7 s). The laser beam was widened by lenses and shaped by an aperture to yield approximately homogenous illumination of a quadratic area of 1 cm^2 matching the width of the used optical cuvette. Pulse intensities were determined using a Nova-Laser-Power/ Energy-Monitor, measuring head PE10 (Ophir Optronics).

To avoid saturation of the detector system caused by the strong prompt fluorescence of the sample during laser excitation, a gated photomultiplier was used (Hamamatsu R2066; PMT

[a] Determined experimentally

Gated Socket Assembly C1392-55; anode voltage, 1000 V; anode resistor, 2.2 kΩ; gating voltage, 240 V applied from 7µs before to 3 µs after the Laser flash). Scattered Laser light was blocked by a combination of two long-pass filters (LINOS Photonics, DT-red and DT-magenta with cut-off wavelength 600 and 632 nm, respectively). After amplification (Tektronix AM502, band width of 300 kHz), the signal was sampled at 1 MHz by a 12-bit PC-card (ADLINK, PCI 9812). The time between flashes was 700 ms.

2.3.8 EPR Spectroscopy

EPR spectroscopy was performed in the laboratory of Dr. Friedhelm Lendzian (TU-Berlin) on an X-band Bruker ESP 300E spectrometer equipped with a helium cryostat (Oxford) (microwave frequency of 9.6 GHz). Further conditions: Six-line Mn^{II} (temperature, T = 20 K) and multiline signals (T = 8 K), microwave power (MP) 20 mW, modulation frequency (MF) 100 kHz, modulation amplitude (MA) 25 Gauss; Tyr_D^{ox} radical, T = 80 K, MP = 100 µW, MF = 12 KHz, MA = 2 Gauss. The multiline signal was generated by strong illumination (400 nm < **λ** < 750 nm) of samples in acrylic-glass holders for 2 min in a dry-ice/ethanol bath at ~ 200 K.

2.3.9 XANES and EXAFS measurements

XAS at the Mn K-edge was performed at the BESSY-II (Berlin) at the double-crystal monochromator beamline KMC-1 (photon flux around 6.5 keV attenuated to ~10^9 photons $s^{-1}mm^{-2}$, spot size on the sample about 1x1 mm^2) using an energy-resolving single-element germanium detector (Canberra, active area ~1 cm^2) for detection of the excited X-ray fluorescence, digital signal processing for pulse detection (DXP from XIA), and a liquid-helium cooled cryostat with height-adjustable sample rod (Oxford). To avoid absorption of the X-ray fluorescence by an intermitting gas layer and to maximize the solid angle for X-ray detection, the detector head was included in the thermal insulation vaccum of the cryostat. The resulting sample-detector distance was ~3 cm (center of sample to center of detector element). A chromium foil (10 µm) in front of the detector largely suppressed scattered X-rays so that detector saturation was kept well below a level of 20 % at a total count rate of ~10^5 s^{-1}. The pulses at the DXP output, which are proportional to the Mn Kα fluorescence yield, were detected in a single-channel analyzer window of ~150 eV width centered around 5900 eV and counted by the beamline electronics. XAS measurements were performed at 10 K, by scanning of the monochromator (Si111 crystals, scan range 6500-7200 eV). X-ray fluorescence signals were corrected for detector saturation using the total count rate

delivered by the DXP. The energy axis of the XAS spectra was corrected according to the position of the narrow pre-edge peak at 6543.3 eV of a $KMnO_4$ standard measured simultaneously in absorption mode (Iuzzolino et al., 1998; Dau et al., 2004). Spectra were normalized as described in (Dau et al., 2003) and up to 15 scans, each performed on a separate spot of the samples, were averaged. The given K-edge energies were determined by the "integration method" outlined in (Dau et al., 2003). XAS samples were prepared by filling ~20 µL of pellets of previously heated, photoactivated PSII membrane samples (see figure legends), which previously had been collected by centrifugation, into acrylic glass sample holders covered on one side with kapton foil. Subsequently the samples were frozen in liquid nitrogen. The Mn concentration in the XAS samples was ~200 µM in control PSII membrane samples and lower in the photoactivated preparations. EXAFS spectra were extracted and simulations were performed by least-squares curve-fitting of k^3-weighted oscillations in the *k*-space as previously described (Dau et al., 2003) using phase functions calculated by Feff-7 (Zabinsky et al., 1995). For further details of the applied procedure of EXAFS data analysis see (Dau et al., 2003; Haumann et al., 2005b).