*Biochimica et Biophysica Acta*, 936 (1988) 259–268
Elsevier



BBA 42870

# Location of manganese atoms in Photosystem II studied by EPR power saturation of Signal $II_{slow}$

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(Received 29 March 1988)
(Revised manuscript received 22 July 1988)

Key words: Photosystem II; EPR; Manganese; Signal II; Oxygen evolution; (*Spinach chloroplast*)

**Continuous-wave power saturation profile of EPR Signal $II_{slow}$ ($II_s$) at 20 K has been used to probe location of the manganese atoms in spinach Photosystem II (PS II) particles. There were at least two classes of the bound manganese atoms; two of the four atoms per PS II were loosely associated and the others were rather tightly associated. Both the classes of manganese were observed to interact magnetically with Signal $II_s$ species ($D^+$). The effectiveness of the loosely bound manganese on the electron-spin relaxation of $D^+$ was little different from that of the tightly bound manganese, suggesting that these two classes of manganese exist at similar distances to $D^+$. Illumination of the PS-II particles resulted in relief of the saturation. It was indicated that the relaxation of $D^+$ was affected by the oxidation state of the manganese complex and also by the state of other component(s) of PS II. The effects of a free-radical-relaxing agent dysprosium (Dy) ion and its complex with EDTA were investigated in the presence and absence of the bound manganese atoms. Removal of the loosely bound manganese increased the accessibility of $Dy^{3+}$ to $D^+$ and decreased that of Dy-EDTA$^-$, whereas further removal of manganese had little effect on the accessibilities of these agents. These results suggest that the loosely bound manganese atoms exist at exposed sites on the membrane surface, whereas the other manganese atoms are buried in the membrane.**

## Introduction

Photosynthetic oxidation of water to molecular oxygen is established to be catalyzed by two- or four-manganese cluster located on the donor side of PS II (for review, see Ref. 1). The oxidizing power generated by photooxidation of the PS-II reaction center chlorophyll P-680 is transferred to the oxygen-evolving complex via the intermediate electron carrier, Z [2–4]. The oxygen-evolving complex goes through five redox states denoted $S_0$–$S_4$ during the water oxidation [5]. On the donor side of PS II, there is another component D, which can reduce the oxygen-evolving complex in the $S_2$ and $S_3$ states and is unusually stable in the oxidized form [6,7]. Illumination of PS II causes oxidation of these donors, D and Z, and induces the respective EPR signals, called Signal $II_s$ and $II_{vf}$. These signals have an identical lineshape but can be distinguished by their very fast (Signal $II_{vf}$) and slow (Signal $II_s$) decay kinetics after the

Abbreviations: Chl, chlorophyll; DCMU, 3-(3,4)-dichlorophenyl-1,1-dimethylurea; EPR, electron paramagnetic resonance; Mes, 4-morpholineethanesulfonic acid; PS II, Photosystem II; Signal $II_s$, $II_f$ and $II_{vf}$, EPR Signal $II_{slow}$, $II_{fast}$ and $II_{very\ fast}$, respectively.

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illumination. The decay of Signal $II_{vf}$ is slowed down by inhibition of the oxygen evolution, and the slowly decaying form of Signal $II_{vf}$ is called Signal $II_f$. Up to now, the physiological role of D has been scarcely understood. However, recent EPR studies suggested involvement of D in stabilization of the manganese complex and in the photoactivation process of oxygen evolution [8,9]. Molecular origins of D and Z are presumably the same and recently have been ascribed to tyrosine residues of the reaction center polypeptides of PS II by EPR studies with cyanobacteria [10,11].

The three peripheral polypeptides with molecular masses of 33, 24 and 18 kDa are assumed to be involved in the oxygen evolution [12–18]. One molecule each of these polypeptides and four manganese atoms per PS-II center were associated with the PS-II particles prepared from spinach chloroplasts [19,20]. After removal of these polypeptides by $CaCl_2$ or urea treatment, two of the four manganese atoms were easily released with concomitant loss of the oxygen evolution activity [16–18]. The binding of the two manganese atoms seems to be stabilized by the peripheral 33 kDa polypeptide, whereas the other two atoms are rather stably bound to PS II without the polypeptide. When the PS-II particles were subjected to trypsin or chymotrypsin, the binding of the manganese atoms showed different susceptibilities to the proteinases between two classes of the manganese [21]. These observations suggest that the manganese atoms bound to PS II are functionally and structurally heterogeneous. The two loosely bound manganese atoms interacting with the 33 kDa polypeptide are directly related to the oxygen evolution activity, whereas the involvement of the other two manganese atoms in the oxygen evolution is still an open question.

The microwave-power saturation behavior of an electron carrier in the paramagnetic state can be a sensitive probe for the structural organization around the carrier [22,23]. The access of a paramagnetic species to the electron carrier will cause a spin–spin interaction between them, which results in a change of the spectral lineshape of the carrier and/or relief from the power saturation. The effects of the paramagnetic species will be determined by their magnetic properties and by their spatial relationship. The saturation behavior of Signal $II_f$ was affected by treatments which perturb binding of manganese, suggesting the magnetic interaction between manganese and $Z^+$ [24–26]. Rutherford et al. [27] reported that Signal $II_s$ in the low-fluorescent mutant of *Scenedesmus*, which contained reduced amount of manganese, was more easily saturated than that in the wild-type. De Groot et al. [28] showed that the spin-lattice relaxation rates of $D^+$ and $Z^+$ were affected by the redox state of the manganese complex by the electron spin-echo study. By analyzing the effects of a free-radical relaxing agent, dysprosium (Dy) ion, and its complex with EDTA [22,29–32] on the power saturation of Signal $II_s$ in the PS-II particles, we showed that the peripheral polypeptides are bound on the membrane surface near $D^+$, and that the binding site of the 24 and 18 kDa polypeptides on the membrane is negatively charged, whereas that of the 33 kDa polypeptide is rather rich in positive charges [33,34].

In the present study, spin–spin interaction between $D^+$ and the bound manganese was studied by analyzing the continuous-wave power saturation of Signal $II_s$ after quantitative removal of manganese. The effects of illumination at low temperature and the added Dy ion and Dy-EDTA complex on the saturation of Signal $II_s$ were also studied. Manganese atoms and the above mentioned relaxing agents affected the electron-spin relaxation of $D^+$ as intrinsic and extrinsic paramagnetic species, respectively. Organization of the manganese complex and its spatial relationships to D and Z are discussed on the basis of the recent development of structural studies of the photosynthetic reaction centers.

## Materials and Methods

Oxygen-evolving PS-II particles were prepared from spinach chloroplasts according to Kuwabara and Murata [13]. The particles were suspended in medium A (0.3 M sucrose, 25 mM Mes-NaOH (pH 6.5), 10 mM NaCl) containing 30% (w/v) glycerol and stored in liquid nitrogen until use. The particles were thawed and washed by centrifugation at $35000 \times g$ for 15 min with medium A and were designated as untreated PS-II particles. The $CaCl_2$ washing which removes the peripheral

polypeptides of 33, 24 and 18 kDa was carried out essentially as described by Ono and Inoue [15]. The stored PS-II particles were thawed and suspended in medium A containing 1.2 M $CaCl_2$ at about 0.2 mg Chl/ml. After incubation at 4°C for 20 min, the suspension was centrifuged at 35000 $\times g$ for 15 min. The resultant pellet was washed once with medium A and designated as 'fresh' $CaCl_2$-washed PS-II particles. To remove manganese from the $CaCl_2$-washed PS-II particles, the particles were incubated in medium A (0.2 mg Chl/ml) at 4°C in darkness for various periods. The particles were then precipitated by centrifugation, and the resultant pellet was washed once with medium A. The $CaCl_2$-washed particles which were incubated in medium A for more than 8 h were designated as 'aged' $CaCl_2$-washed PS-II particles. To thoroughly remove manganese and the peripheral polypeptides, the PS-II particles were suspended in a medium containing 0.8 M Tris-HCl (pH 8.5), 0.3 M sucrose, 10 mM NaCl and 0.5 mM EDTA at 0.2 mg Chl/ml. After incubation at 4°C for 20 min, the suspension was centrifuged at 35000 $\times g$ for 15 min. The resultant pellet was then washed with medium A and designated as Tris-treated PS-II particles.

For EPR measurement, unless otherwise noted, the PS-II particles were suspended in medium A containing 30% glycerol at 4 mg Chl/ml. The suspension was transferred into a quartz EPR tube, incubated at 4°C for 15 min in darkness and frozen in liquid nitrogen. In some experiments, the dark-adapted sample in the EPR tube was illuminated at 200 K for 2 min in a mixture of ethanol and solid $CO_2$ using a 650 W projector lamp. In the experiments with $DyCl_3$ or Dy-EDTA, the PS-II particles were mixed with one of these agents and incubated at 4°C for 15 min in darkness before frozen in liquid nitrogen. Dy-EDTA was obtained by chelating $DyCl_3$ with EDTA (pH of the solution was adjusted to 6.0–6.7.). The first derivative EPR spectra were recorded at 20 ± 0.5 K using a Bruker ER-200 X-band spectrometer fitted with an Oxford Instruments ESR-900 liquid-helium cryostat.

Manganese contents of the samples were determined by an EPR measurement after treatment with 0.5 M HCl as described by Yocum et al. [26]. Oxygen-evolution activity was assayed in a medium containing 300 mM sucrose, 25 mM Mes-NaOH (pH 6.0) and 200 mM NaCl with 0.3 mM phenyl-*p*-benzoquinone as an electron acceptor using a Clark-type oxygen electrode at 25°C.

Chlorophyll was determined according to Arnon [35]. The molar ratio of one PS-II reaction center to 220 chlorophyll molecules was assumed for the PS-II particles used [19].

## Results

### *Removal of manganese*

Both the untreated and the fresh $CaCl_2$-washed particles contained about four manganese atoms per 220 Chl. The fresh $CaCl_2$-washed particles retained about a quarter of the oxygen-evolution activity of the untreated sample. In the $CaCl_2$-washed particles incubated in medium A, the oxygen-evolution activity was gradually lost with the release of manganese (not shown). After the incubation for 8 h, the activity completely disappeared and about half the amount of manganese was released from the particles. Consequently, the aged $CaCl_2$-washed PS-II particles contained about two manganese atoms per 220 Chl. On the other hand, almost all manganese was removed by the treatment with alkaline Tris and EDTA. These results summarized in Table I well agree with the previous reports [12,13,16–18].

### *Power-saturation analysis*

In Fig. 1, Signal $II_s$ spectra in the untreated dark-frozen PS-II particles at 20 K are shown as a function of the applied microwave power. The

TABLE I

MANGANESE CONTENTS AND OXYGEN-EVOLUTION ACTIVITIES OF THE FOUR TYPES OF PS-II PARTICLES

| PS-II particles | Mn content [a] (atoms/220 Chl) | $O_2$ evolution ($\mu$mol·(mg Chl·h)$^{-1}$) |
|---|---|---|
| Untreated | 3.83 ± 0.05 | 486 |
| $CaCl_2$-washed | | |
| fresh | 3.79 ± 0.10 | 131 |
| aged | 2.01 ± 0.18 | 0 |
| Tris-treated | 0.36 ± 0.14 | 0 |

[a] Data are average ± S.D. of five and four separate determinations for the untreated sample and the other samples, respectively.

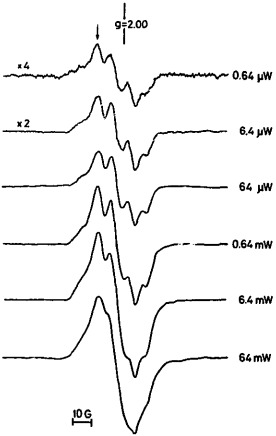


Fig. 1. Dependence of Signal $II_s$ spectrum in the untreated PS-II particles on incident microwave power. The trace at 0.64 μW was average of two scans. The amplitude of the lowest field peak marked by the arrow was used for analysis of the saturation behavior. EPR conditions: temperature, 20 K; microwave frequency, 9.6 GHz; modulation amplitude, 4 G; scanning rate, 2 G/s; time constant, 160 ms.

spectral lineshape with the hyperfine structure did not change in the microwave-power range smaller than 1 mW. Apparent line broadening of the EPR signal occurred at the higher microwave powers. Fig. 2 shows the power saturation profile of Signal $II_s$ in the untreated PS-II particles. The power saturation profile was analyzed by plotting the signal amplitude ($S$) divided by $\sqrt{P}$ against the microwave power ($P$), both on logarithmic scales. This type of plot is expected to give a straight line parallel to the abscissa in the range of microwave power at which no saturation takes place, and to go downward as the saturation begins. At the higher microwave powers, it gives a straight line with a slope which is determined by the degree of inhomogeneous broadening [30,36]. This plot is helpful to obtain an empirical half-saturation parameter $P'_{1/2}$ which indicates the microwave power at which $S/\sqrt{P}$ is one-half of its

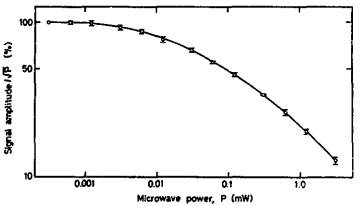


Fig. 2. Microwave power saturation profile of Signal $II_s$ in the untreated PS-II particles. Vertical bars represent S.D. for five separate measurements. EPR conditions are the same as in Fig. 1.

unsaturated value *. $P'_{1/2}$ value of Signal $II_s$ obtained for the untreated PS-II particles was 0.096 ± 0.008 mW (average ± S.D. of five experiments) at 2 K.

*Effects of bound manganese*

In Fig. 3, the spectra of Signal $II_s$ in the fresh $CaCl_2$-washed particles and Tris-treated particles at low and high microwave powers are displayed. At a nonsaturating low microwave power, the signal amplitudes in the fresh $CaCl_2$-washed particles and the Tris-treated particles were the same as that in the untreated particles on the basis of the chlorophyll concentration. This shows that the amounts of $D^+$ were the same in these preparations. At the higher microwave power, however, the amplitude in the fresh CaCl-washed particles was significantly larger than that in the Tris-treated particles. The power-saturation profiles of Signal

* The parameter $P'_{1/2}$ is related to the theoretical half-saturation parameter $P_{1/2}$, by the multiplicative correction factor $k$, i.e., $P'_{1/2} = kP_{1/2}$. The theoretical saturation parameter $P_{1/2}$ is the microwave power corresponding to the microwave magnetic field $H_{1/2}$ at which the saturation condition $\gamma H_{1/2}(T_1T_2)^{1/2} = 1$ is met, where $\gamma$ is the gyromagnetic constant, and $T_1$ and $T_2$ are the spin-lattice relaxation time and the spin-spin relaxation time, respectively. $k$ is a known function of a parameter $a$ or the ratio of the Lorentzian line width to the Gaussian line width, $\Delta H_L/\Delta H_g$, which measures the degree of inhomogeneous broadening [37]. In the present definition of the parameters, $k$ ranges from 1/3 for the completely inhomogeneous case ($a = 0$) to 1 for the completely homogeneous case ($a \gg 1$).

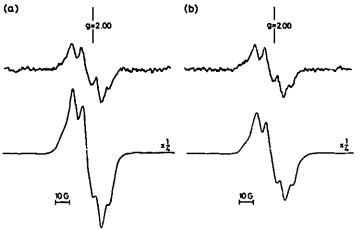


Fig. 3. Spectra of Signal $II_s$ in the two types of PS-II particle recorded at different microwave powers. (a) Fresh $CaCl_2$-washed PS-II particles; (b) Tris-treated PS-II particles. The applied microwave powers were 0.64 μW (upper trace) and 0.64 mW (lower trace). EPR conditions are the same as in Fig. 1.

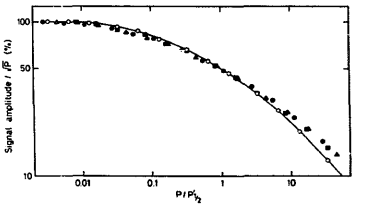


Fig. 5. Saturation profiles of Signal $II_s$ in the four types of PS-II particle, plot of the signal amplitude/$\sqrt{P}$ versus $P/P'_{1/2}$. The data points displayed in Figs. 2 and 4 were replotted. $P'_{1/2}$ values of 0.096, 0.0112, 0.072 and 0.038 mW were assumed for the untreated (○——○), fresh $CaCl_2$-washed (●), aged $CaCl_2$-washed (▲) and Tris-treated (■) PS-II particles, respectively.

$II_s$ in the three preparations, which contained different amounts of manganese and no peripheral polypeptides, are shown in Fig. 4. Signal $II_s$ in the aged $CaCl_2$-washed particles was saturated at a microwave power lower than that in the fresh sample. Signal $II_s$ in the Tris-treated particles was saturated at a still lower microwave power. These results suggest that the power saturation of Signal $II_s$ depends on the manganese content of the PS-II particles. This was also confirmed by the result that washing of the PS-II particles with 1 M NaCl, which removes the 24 and 18 kDa polypeptides but does not remove the 33 kDa polypeptide nor manganese, did not affect the saturation profile (not shown).

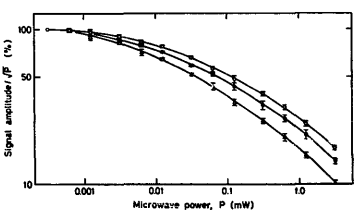


Fig. 4. Microwave power saturation profiles of Signal $II_s$ in the three types of PS-II particles. Fresh $CaCl_2$-washed particles (○); aged $CaCl_2$-washed particles (●); Tris-treated particles (△). Error bars represent S.D. for four separate measurements. EPR conditions are the same as in Fig. 1.

The power saturation profile can be characterized by two parameters: the empirical half-saturation parameter $P'_{1/2}$ (or the theoretical parameter $P_{1/2}$) and the inhomogeneity parameter, $a$. If parameter $a$ is constant, the empirical parameter $P'_{1/2}$ is proportional to the theoretical parameter $P_{1/2}$ (see the previous footnote *), and defines the saturation curve uniquely. To check the difference of parameter $a$, Signal $II_s$ amplitude/$\sqrt{P}$ was replotted against $P/P'_{1/2}$ (Fig. 5). The power saturation curves of Signal $II_s$ in the three preparations, i.e., the fresh and aged $CaCl_2$-washed particles and the Tris-treated particles, showed good agreement with each other in these plottings. This shows that parameter $a$ is constant in these preparations and that the use of the empirical parameter $P'_{1/2}$ is valid in comparing the theoretical parameter $P_{1/2}$ or the electron-spin relaxation rate of $D^+$. On the other hand, the saturation curve plotted against $P/P'_{1/2}$ for the untreated particles was slightly but consistently different from the curves for the other three preparations; the $CaCl_2$ washing and Tris treatment caused the saturation curve to be more inhomogeneous. The difference in the $P'_{1/2}$ value between the untreated and the other preparations seems to reflect the differences in both the parameters $a$ and $P_{1/2}$.

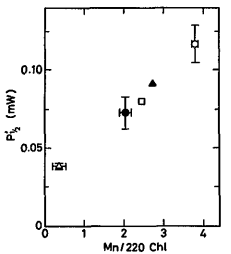


Fig. 6. Dependence of saturation parameter $P'_{1/2}$ on manganese content of the PS-II particles depleted of the peripheral polypeptides. Fresh $CaCl_2$-washed particles (○); $CaCl_2$-washed particles incubated for 2 h (▲) and 4 (□) in medium A; aged $CaCl_2$-washed particles (●); Tris-treated particles (△). Error bars represent S.D. for four separate measurements.

The saturation parameter $P'_{1/2}$ of Signal $II_s$ in the $CaCl_2$-washed and Tris-treated PS-II particles was plotted against the manganese content (Fig. 6). The samples with the intermediate manganese contents between four and two atoms per 220 Chl were prepared by varying the incubation time after the $CaCl_2$ washing. The EPR signal in these preparations had no difference in the parameter $a$ from that in the other preparations that contained no peripheral polypeptides, judging from the saturation profiles plotted against $P/P'_{1/2}$ (not shown). The saturation parameter $P'_{1/2}$ had an approximately linear dependence on the manganese content within the experimental error. The change in $P'_{1/2}$ by removal of the loosely bound manganese was almost the same as that by removal of the tightly bound manganese. This suggests that both classes of the bound manganese atoms interact magnetically with $D^+$ and that their effects on the relaxation of $D^+$ were indistinguishable from each other.

*Effects of illumination at 200 K*

Oxygen evolution in PS II requires successive oxidations of the manganese complex through the intermediate redox states $S_i$ ($i = 0$ to 4) [5]. The $S_2$ state, which gives rise to a multiline EPR signal centered at $g = 2.0$ [38], is accumulated by illumination during freezing with DCMU [39] or by illumination at 200 K [40]. Fig. 7 shows the EPR spectra of the four PS-II preparations illuminated

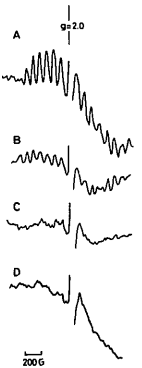


Fig. 7. Difference spectra of the four types of PS-II particle illuminated at 200 K. (A) Untreated PS-II particles; (B) fresh $CaCl_2$-washed particles; (C) aged $CaCl_2$ washed particles; and (D) Tris-treated particles. The samples suspended in a medium containing 0.3 M sucrose, 25 mM Mes-NaOH (pH 6.5), 200 mM NaCl and 30% glycerol were illuminated at 200 K for 2 min. EPR conditions: temperature, 10 K; microwave power, 100 mW; microwave frequency, 9.5 GHz; modulation amplitude, 25 G; scanning rate, 10 G/s; time constant, 200 ms.

at 200 K. The $S_2$-multiline signal clearly appeared in the untreated particles. It was also detected in the fresh $CaCl_2$-washed particles but the signal amplitude was smaller. The $S_2$ signal was not detected in the aged $CaCl_2$-washed particles and in the Tris-treated particles. Such disappearance of the $S_2$ signal was concerted with that of the oxygen-evolution activity.

The illumination of the PS-II particles at 200 K resulted in the relief of the power saturation of Signal $II_s$ (Fig. 8). The effect of the illumination was observed in all the four preparations, and the increase in $P'_{1/2}$ by the illumination in the untreated PS-II particles was larger than the increases in the other preparations which showed reduced or no $S_2$ multiline signal (Table II) **.

** When compared in the theoretical saturation parameter $P_{1/2}$, the effect of the illumination is still larger in the untreated preparation than in the other preparations, because the correction factor $k$ (see the previous footnote *, p. 262) decreases with increase of the inhomogeneity.

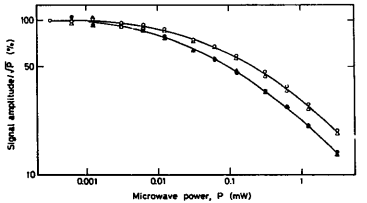


Fig. 8. Effect of illumination at 200 K on the saturation profile of Signal $II_s$ in the untreated PS-II particles. The sample was frozen at 77 K after 15 min dark adaptation at 4°C (●, ▲) or after 2-min illumination at 200 K (○, △). EPR conditions are the same as in Fig. 1.

This shows that not only the oxidizing equivalents accumulated in the manganese complex but that also the redox state of other component(s) in PS II affect the relaxation of $D^+$, although the former have the stronger influence. The saturation profiles plotted against $P/P'_{1/2}$ in all the preparations were not changed by the illumination (not shown), showing that the inhomogeneity was not affected by the illumination.

In the $CaCl_2$-washed and Tris-treated PS-II particles, a 10 G wide, non-structural EPR signal centered at $g = 2.00$, possibly arising from a chlorophyll cation radical [41,42], was produced by the illumination at 200 K and superimposed on Signal $II_s$ (not shown). This signal was more significant in the aged $CaCl_2$-washed and Tris-treated particles than that in the fresh $CaCl_2$-washed particles. However, the contribution of this signal on the lowest field peak of Signal $II_s$, which we used for the data analysis, was small and affected the measurement of the saturation behavior only slightly. The radical species giving rise to this signal may also interact magnetically with $D^+$ in these illuminated samples.

*Effects of the exogenous relaxing agents*

Effects of $Dy^{3+}$ and Dy-EDTA$^-$ on the power saturation of Signal $II_s$ were studied in the fresh and aged $CaCl_2$-washed preparations and Tris-treated preparation in a medium containing 100 mM NaCl. The higher concentration of NaCl was added to minimize the non-specific electrostatic interaction of the paramagnetic ions with the surface charges on the membrane and also to stabilize the bound manganese [17,18]. $P'_{1/2}$ increased with the concentration of the relaxing agents and showed an approximately linear dependence on the concentration [33,34]. Addition of diamagnetic $LaCl_3$ had little effect on the saturation profile of Signal $II_s$ in every preparation. The amount of manganese released from the fresh $CaCl_2$-washed particles by the addition of $LaCl_3$ (2 mM) was less than 10% of the total content under the experimental conditions. The changes in the saturation parameter $P'_{1/2}$ by the relaxing agents are listed in Table III. The effect

TABLE II

FORMATION OF THE $S_2$ MULTILINE SIGNAL AND CHANGE OF $P'_{1/2}$ BY ILLUMINATION AT 200 K IN THE FOUR TYPES OF PS-II PARTICLES

Samples were suspended in a medium containing 0.3 M sucrose, 25 mM Mes-NaOH (pH 6.5), 200 mM NaCl and 30% glycerol, and illuminated at 200 K for 2 min. The amplitude of the $S_2$ multiline signal is taken as the average of peak-to-peak heights of five lines downfield from $g = 2.0$.

| PS-II particles | Relative amplitude of multiline signal | Light-induced change of $P'_{1/2}$ (mW) |
|---|---|---|
| Untreated | 100 | 0.0116 |
| $CaCl_2$ washed | | |
| fresh | 26 | 0.076 |
| aged | nd [a] | 0.048 |
| Tris-treated | nd [a] | 0.053 |

[a] nd, not detected.

TABLE III

CHANGES IN $P'_{1/2}$ BY RELAXING AGENTS $DyCl_3$ AND Dy-EDTA COMPLEX

Samples were suspended in a medium containing 0.3 M sucrose, 25 mM Mes-NaOH (pH 6.5), 100 mM NaCl, 30% glycerol and the relaxing agent.

| PS-II particles | $\Delta P'_{1/2}$ (mw) | |
|---|---|---|
| | 2 mM $DyCl_3$ | 10 mM Dy-EDTA |
| Fresh $CaCl_2$-washed particles | 4.9 | 0.56 |
| Aged $CaCl_2$-washed particles | 12.0 | 0.35 |
| Tris-treated particles | 12.0 | 0.34 |

of $Dy^{3+}$ was larger in the aged $CaCl_2$-washed particles than that in the fresh sample, whereas the effect of Dy-EDTA$^-$ was smaller in the aged $CaCl_2$-washed particles than that in the fresh sample. On the other hand, the Tris-treated particles showed almost the same sensitivities to these agents as the aged $CaCl_2$-washed particles. These results suggest that the surface near $D^+$ in the fresh $CaCl_2$-washed particles has more positive charges than that in the aged sample, and that the charge distribution on the surface near $D^+$ in the aged $CaCl_2$-washed particles is similar to that in the Tris-treated particles.

## Discussion

In the present work, the microwave-power saturation profile of Signal $II_s$ at 20 K was studied to get information on the location of manganese atoms in PS II. With the release of manganese, Signal $II_s$ was saturated by lower microwave energy. The empirical half-saturation parameter $P'_{1/2}$ decreased equally by removal of the loosely bound manganese and by that of the tightly bound manganese. The degrees of inhomogeneous broadening in the PS-II preparations used for the analysis of the manganese effects were almost the same. These results suggest that the electron-spin relaxation of $D^+$ is accelerated by the spin–spin interactions with these manganese atoms. If oxidation states of the manganese atoms were equal in the $CaCl_2$-washed sample under the dark condition, both classes of manganese exist at similar distances to $D^+$. Oxidation states of the manganese atoms corresponding to each S state have been a matter of discussion. However, a recent analysis on X-ray absorption near-edge structure suggested that almost all the oxygen-evolving centers under the dark condition were in the $S_1$ state, which was ascribed to the redox states of Mn (III, III, III, III) [43]. This supports our assumption.

Signal $II_s$ in the PS-II preparations treated with $CaCl_2$ or alkaline Tris showed more inhomogeneous saturation than that in the untreated particles. The inhomogeneous broadening of an EPR signal comes from the interaction with a species whose EPR relaxation is slow compared with that for the signal species [37]. High-potential form of cytochrome $b_{559}$ is converted to the low-potential form and oxidized after the $CaCl_2$ washing or the Tris treatment [44]. The oxidized form of cytochrome $b_{559}$ is paramagnetic and possible to interact magnetically with $D^+$. Hence, the redox change of cytochrome $b_{559}$ may be responsible for the difference in the inhomogeneity between the untreated PS-II particles and the other preparations.

The relief of the power saturation of Signal $II_s$ by the illumination of the untreated and fresh $CaCl_2$-washed PS-II particles, which showed the $S_2$ multiline signal, suggests that the electron-spin relaxation of $D^+$ is affected by the redox state of the manganese complex. However, the illumination of the aged $CaCl_2$-washed and Tris-treated particles, which had lower manganese contents and formed no multiline signal, also relieved the power saturation of Signal $II_s$, though to a smaller extent than that of the preparations showing the multiline signal. The effect of the illumination might be due to the interaction of $D^+$ with a free radical, probably a chlorophyll cation [41,42], which is induced by the low-temperature illumination under the conditions in which electron donation from the manganese complex and cytochrome $b_{559}$ to $P680^+$ is prevented [45]. The results obtained here agree with those obtained with electron spin-echo study by de Groot et al. [28], although they did not consider the effects of light-induced paramagnetic species other than manganese as the relaxing species for $D^+$ and $Z^+$. We conclude here that the relaxation of $D^+$ is affected mainly by the manganese complex and also by the other component(s) which become paramagnetic upon illumination.

Recently, we reported that accessibility of $Dy^{3+}$ or Dy-EDTA$^-$ to $D^+$ is different in the untreated NaCl-washed and $CaCl_2$-washed PS-II preparations which have the same manganese contents and different compositions of the peripheral polypeptides [33,34]. It was concluded that the binding site of 24 kDa and/or 18 kDa polypeptides on the membrane surface of PS II was negatively charged and the binding site of the 33 kDa polypeptide was rather rich in positive charges. In the present work, we studied the accessibilities of $Dy^{3+}$ and Dy-EDTA$^-$ to $D^+$ in the PS-II preparations which lacked all the peripheral polypeptides but contained different amounts of manganese. The results obtained here show that the positive surface

charges near $D^+$, probably exposed by the removal of the 33 kDa polypeptide, in the fresh $CaCl_2$-washed particles are decreased by the removal of the two loosely bound manganese atoms, but are not affected by the further removal of the two tightly bound manganese atoms. The loosely bound manganese seems to be exposed on the membrane surface and to affect accessibilities of the relaxing agents, whereas the other manganese seems to exist in the interior of the membrane and to give little effect on the surface charge. This conclusion is consistent with the results obtained by the proteinase treatment of the PS-II particles [21] or by surface-enhanced Raman scattering spectroscopy [46].

Several lines of evidences have been accumulated that indicate the two functionally different classes of manganese in the oxygen-evolution system [1,16–18]. In four manganese models of the water-oxidation cycle [47–50], two of the four manganese atoms are involved in ligating water molecules and undergo a redox transition different from that of the other two atoms. These classes of manganese seem to correspond to the exposed and buried atoms shown by the present work, and may be situated at similar distances to $D^+$. Relatively weak interactions of the manganese atoms with $D^+$ suggest a distance of more than several Ångstroms (Å) between these species.

Assuming that the four manganese atoms cooperate in oxygen evolution, distances between the manganese atoms seem to be a few Å. Data on extended X-ray absorption fine structure indicated that there is at least one binuclear manganese center per PS II, in which Mn–Mn distance is approx. 2.7 Å [51]. On the other hand, tyrosine residues presumably corresponding to Z and D are symmetrically located on the respective transmembrane helixes III of the D1 and D2 polypeptides [11,52–55]. By analogy with the X-ray structure of bacterial reaction centers [56,57], the distance between these tyrosine residues can be estimated at about 30 Å. The weak interaction of manganese with $Z^+$ suggests a rather long distance between them [26]. This situation is similar to the case of D reported here. From these observations, we propose a structural model in which the manganese cluster lies near midway between Z and D. The peripheral 33 kDa polypeptide may share coordination sites of the exposed manganese atoms with the reaction center polypeptides, whereas the other manganese atoms may coordinate only to the reaction center polypeptides (for discussion on interactions between manganese and the peripheral polypeptides, see refs. 58 and 59). Indirect evidence for the association of manganese with D1 and/or D2 polypeptides have been accumulated [53,60], which supports our proposition on the structure of the oxygen-evolving complex.

## Acknowledgements

We thank Dr. Y. Fujita, National Institute for Basic Biology (NIBB), for helpful discussions. Thanks are also due to Mr. H. Hattori and Mr. H. Kojima for their help in EPR measurements performed at the Center for Chemical and Physical Analysis of NIBB. This work was supported by a Cooperative Research Program of NIBB, and by Grants-in-Aid for Research on Priority Areas of 'Bioenergetics' from the Ministry of Education, Science and Culture of Japan.

## References

1 Amesz, J. (1983) Biochim. Biophys. Acta 726, 1–12.
2 Bouges-Bocquet, B. (1980) Biochim. Biophys. Acta 594, 85–103.
3 Babcock, G.T., Blankenship, R.E. and Sauer, K. (1976) FEBS Lett. 61, 286–289.
4 Boska, M., Sauer, K., Buttner, W.J. and Babcock, G.T. (1983) Biochim. Biophys. Acta 722, 327–330.
5 Kok, B., Forbush, B. and McGloin, M. (1970) Photochem. Photobiol. 11, 457–475.
6 Babcock, G.T. and Sauer, K. (1973) Biochim. Biophys. Acta 325, 485–503.
7 Velthuys, B.R. and Visser, J.W.M. (1975) FEBS Lett. 55, 109–112.
8 Styring, S. and Rutherford, W. (1987) Biochemistry 26, 2401–2405.
9 Nugent, J.H.A., Demetriou, C. and Lockett, C.J. (1987) Biochim. Biophys. Acta 894, 534–542.
10 Barry, B.A. and Babcock, G.T. (1987) Proc. Natl. Acad. Sci. USA 84, 7099–7103.
11 Debus, R.J., Barry, B.A., Babcock, G.T. and McIntosh, L. (1988) Proc. Natl. Acad. Sci. USA 85, 427–430.
12 Yamamoto, Y., Doi, M., Tamura, N. and Nishimura, M. (1981) FEBS Lett. 133, 265–268.
13 Kuwabara, T. and Murata, N. (1982) Plant Cell Physiol. 23, 533–539.
14 Åkerlund, H.-E., Jansson, C. and Andersson, B. (1982) Biochim. Biophys. Acta 681, 1–10.

15 Ono, T. and Inoue, Y. (1984) FEBS Lett. 164, 255–260.
16 Ono, T. and Inoue, Y. (1984) FEBS Lett. 168, 281–286.
17 Miyao, M. and Murata, N. (1984) FEBS Lett. 170, 350–354.
18 Kuwabara, T., Miyao, M., Murata, T. and Murata, N. (1985) Biochim. Biophys. Acta 806, 283–289.
19 Murata, N., Miyao, M., Omata, T., Matsunami, T. and Kuwabara, T. (1984) Biochim. Biophys. Acta 765, 363–369.
20 Yamamoto, Y., Tabata, K., Isogai, Y., Nishimura, M., Matsuura, K. and Itoh, S. (1984) Biochim. Biophys. Acta 767, 493–500.
21 Isogai, Y., Yamamoto, Y. and Nishimura, M. (1985) FEBS Lett. 187, 240–244.
22 Case, G.D. and Leigh, J.S. (1976) Biochem. J. 160, 769–783.
23 Ohnishi, T., LoBrutto, R., Salerno, J.C., Bruckner, R.C. and Frey, T.G. (1982) J. Biol. Chem. 25, 14821–14825.
24 Warden, J.T., Blankenship, R.E. and Sauer, K. (1976) Biochim. Biophys. Acta 423, 462–478.
25 Yocum, C.F. and Babcock, G.T. (1981) FEBS Lett. 130, 99–102.
26 Yocum, C.F., Yerkes, C.T., Blankenship, R.E., Sharp, R.R. and Babcock, G.T. (1981) Proc. Natl. Acad. Sci. USA 78, 7507–7511.
27 Rutherford, A.W., Seibert, M. and Metz, J.G. (1988) Biochim. Biophys. Acta 932, 171–176.
28 De Groot, A., Plijter, J.J., Evelo, R., Babcock, G.T. and Hoff, A.J. (1986) Biochim. Biophys. Acta 848, 8–15.
29 Antholine, W.E., Hyde, J.S. and Swartz, H.M. (1978) J. Magn. Reson. 29, 517–522.
30 Blum, H., Leigh, J.S. and Ohnishi, T. (1980) Biochim. Biophys. Acta 626, 31–40.
31 Blum, H., Cusanovich, M.A., Sweeney, W.V. and Ohnishi, T. (1981) J. Biol. Chem. 256, 2199–2206.
32 Hyde, J.S. and Rao, K.V.S. (1978) J. Magn. Reson. 29, 509–516.
33 Itoh, S., Isogai, Y., Tang, X.-S. and Satoh, K. (1987) in Progress in Photosynthesis Research (Biggins, J., ed.), Vol. I, pp. 483–486, Martinus Nijhoff Publishers.
34 Isogai, Y., Nishimura, M. and Itoh, S. (1987) Plant Cell Physiol. 28, 1493–1499.
35 Arnon, D.I. (1949) Plant Physiol. 24, 1–15.
36 Rupp, H., Rao, K.K., Hall, D.O. and Cammack, R. (1978) Biochim. Biophys. Acta 537, 255–269.
37 Castner, T.G. (1959) Phys. Rev. 115, 1506–1515.
38 Dismukes, G.C. and Siderer, Y. (1981) Proc. Natl. Acad. Sci. USA 78, 274–278.
39 Hansson, Ö. and Andreasson, L.-E. (1982) Biochim. Biophys. Acta 679, 261–268.
40 Brudvig, G.W., Casey, J.L. and Sauer, K. (1983) Biochim. Biophys. Acta 723, 366–371.
41 Visser, J.W.M., Rijgersberg, C.P. and Gast, P. (1977) Biochim. Biophys. Acta 460, 36–46.
42 Okamura, M.Y., Feher, G. and Nelson, N. (1982) in Photosynthesis: Energy Conversion by Plants and Bacteria (Govindjee, ed.), pp. 195–272, Academic Press, New York.
43 Kusunoki, M., Ono, T., Inoue, Y., Ohyanagi, H., Ishiguro, T. and Matsushita, T. (1988) in Proc. Annu. Meeting Japn. Soc. Plant Physiol., Osaka, pp. 116–117, The Japanese Society of Plant Physiologists.
44 Matsuura, K. and Itoh, S. (1985) Plant Cell Physiol. 26, 537–542.
45 De Paula, J.C., Innes, J.B. and Brudvig, G.W. (1985) Biochemistry 24, 8114–8120.
46 Seibert, M. and Cotton, T.M. (1985) FEBS Lett. 182, 34–38.
47 Critchley, C. and Sargeson, A.M. (1984) FEBS Lett. 177, 2–5.
48 Saygin, Ö. and Witt, H.T. (1987) Biochim. Biophys. Acta 893, 452–469.
49 Kusunoki, M. (1983) in The Oxygen-evolving System of Photosynthesis (Inoue, Y., Crofts, A.E., Govindjee, Murata, N., Renger, G. and Satoh, K., eds.), pp. 165–173, Academic Press, Tokyo.
50 Kambara, T. and Govindjee (1985) Proc. Natl. Acad. Sci. USA 82, 6119–6123.
51 Yachandra, V.K., Guiles, R.D., McDermott, A., Britt, R.D., Dexheimer, S.L., Sauer, K. and Klein, M.P. (1986) Biochim. Biophys. Acta 850, 324–332.
52 Takahashi, Y. and Styring, S. (1987) FEBS Lett. 223, 371–375.
53 Ikeuchi, M. and Inoue, Y. (1988) Plant Cell Physiol. 29, 695–705.
54 Michel, H., Weyer, K.A., Gruenberg, H., Dunger, I., Oesterhelt, D. and Lottspeich, F. (1986) EMBO J. 5, 1149–1158.
55 Trebst, A. (1986) Z. Naturforsch. 41c, 240–245.
56 Deisenhofer, J., Epp. O., Miki, K., Huber, R. and Michel, H. (1985) Nature 318, 618–624.
57 Allen, J.P., Feher, G., Yeates, T.O., Komiya, H. and Rees, D.C. (1987) Proc. Natl. Acad. Sci. USA 84, 6162–6166.
58 Yamamoto, Y., Shinkai, H., Isogai, Y., Matsuura, K. and Nishimura, M. (1984) FEBS Lett. 175, 429–432.
59 Yamamoto, Y. (1988) J. Biol. Chem. 263, 497–500.
60 Metz, J.G., Wong, J. and Bishop, N.I. (1980) FEBS Lett. 114, 61–66.