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EPR study of charge recombination via D^+ in the S_2 state of oxygen evolving Photosystem II

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The deactivation process of the S_2 state has been studied by EPR at 4.5 K by means of storage of 195 K illuminated Photosystem II (PS II) membranes between 77 and 273 K. The decay rate of Signal II_s intensity by recombination of D^+ with Q_A^- has an activation energy of 2.0 kJ/mol below 180 K. The linear relation between the decay of multiline and recovery of Signal II_s intensities shows a direct electron transfer between the manganese cluster and D. The activation energy for this process is estimated to be 16.6 kJ/mol below 220 K. Above 250 K, Q_A^- and Q_B^- contribute to the deactivation of the S_2 state with slower rates and a higher activation energy of 46.6 kJ/mol. The relation between the $g = 4.1$ signal decay and Signal II_s recovery in the Cl^--depleted PS II suggests that the $g = 4.1$ signal can be ascribed to the same manganese cluster which yielded the multiline in ordinary PS II.

Introduction

Since the first discovery of Sig. II in Photosystem II [1], many authors have studied the properties of this radical species. Sig. II_{vf} in the oxygen-evolving Photosystem II was assigned to Z^+, the donor to P-680 [2], which turned out to produce Sig. II_f with a slower decay characteristic by Tris-treatment [3]. On the other hand, Sig. II_s (stable or slowly decaying in the dark) [4] has been ascribed to D^+, which is located also at the donor side of P-680 and which function has not yet been clarified. The radical species, once identified as a plastoquinone cation [5], has recently been ascribed to a tyrosin radical by Barry and Babcock [6] from their study of isotope substitution.

The properties of EPR Sig. II_s has recently been extensively studied by three groups [7–9]. Styring and Rutherford [7] proved that D^+ is reduced by the S_0 state in the oxygen-evolving system during dark adaptation, while its reduced form D works as a reductant to the higher S-states, S_2 and S_3, at a physiological condition. Nugent et al. [8] has also proved from their EPR studies of PS II membranes stored at 77 K by a thawing method that D^+ and Q_A^- recombine at 77 K. D and S_2 recombine at 273 K, accompanied by recovery in Sig. II_s and decay in the multiline [10]. Their results agree with our previous report [9] in which we have obtained similar results on Sig. II_s decay during storage at 77 K and its recovery at the lower temperature 210 K accompanied by disappearance of the multiline signal. The multiline signal was first observed by Dismukes and Siderer [10] at the low temperature of 8 K and has been used as a monitor of the Kok's S_2 state [11] by many workers. Later, a $g = 4.1$ signal was observed in the PS II illuminated at lower temperature [12,13], which converted to the multiline above 180 K. On the other hand, the same signal could be produced in the Cl^--depleted [14] and NH_2OH-treated samples [15] at 195 K. The origin of this signal has been ascribed to a manganese cluster similar to that for the multiline by de Paula et al. [16].

Our main purpose is to study the role of D^+ as a charge carrier in the oxygen evolving photosystem II in connection with Kok's S states. In this paper we have quantified the relation between Sig. II_s recovery and multiline decay in the illuminated samples with various decay ratios of Sig. II_s after various storage periods, to

Abbreviations: Mops, 4-morpholinepropanesulfonic acid; Mes, 4-morpholineethanesulfonic acid; Hepes, 4-(2-hydroxyethyl-1-piperazineethanesulfonic acid; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Chl, chlorophyll; Q_A, primary quinone acceptor; Q_B, secondary quinone acceptor; PS II, Photosystem II.

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find any stable intermediate between them. The maximum intensity of the multiline can be obtained by repeated cycles of dark adaptation at 273 K and illumination at 195 K [17]. At the same time we have studied characteristics of decay and recovery of Sig. II_s in more detail by varying storage temperatures and time. The temperature dependence of rate of charge recombination will be discussed in connection with activation energies. The decay rate of the multiline intensity immediately after illumination will be compared with that obtained after three weeks storage of the illuminated sample at 77 K in order to investigate the role of D as a reductant of the S_2 state.

We have also compared the experimental results in the Cl^--depleted and Br^--substituted samples with those in the ordinary PS II particles. The $g = 4.1$ signal in the Cl^--depleted sample has been observed without conversion to the multiline to compare with the characteristics of charge recombination in the control PS II membranes.

Materials and Methods

O_2-evolving PS-II membranes (400–530 μmol O_2/mg Chl · h) were prepared from spinach by the method of Kuwabara and Murata [18], and finally suspended in the buffer medium containing 0.2 M sucrose, 20 mM NaCl and 20 mM Mops-NaOH (pH 6.9). Cl^- depletion and Br^- substitution were performed as described in Ref. 14. The PS-II membranes, thus treated, were finally suspended in 400 mM sucrose, 40 mM Hepes-NaOH (pH 7.5) supplemented with 50 mM Na_2SO_4 and NaBr for Cl^- depletion and Br^- substitution, respectively. 50 vol.% glycerol was added to all samples. DCMU, where used, was added to a final concentration of 100 μM (1% dimethyl sulfoxide) in darkness at 273 K. After 4 h dark adaptation at 273 K, the samples with 5 mg Chl per ml were subjected to EPR measurement for dark-background subtraction or to storage at 77 K. All treatments were given under dim green light except where noted otherwise.

For EPR measurement, the samples were illuminated for 5 min at 195 K in an ethanol/solid CO_2 bath as previously described [9]. After illumination, the sample was quickly cooled down by dipping in liquid N_2 in the dark. Illumination was repeated in some samples after dark adaptation at 273 K to reach the maximum ratio of the S_2 state. Dark storage prior to EPR measurement at 4.5 K was performed in a pentane bath or a water bath at differently regulated temperatures for variable periods. The multiline signal and Sig. II_s were measured at 4.5 K with a Bruker ESP 300 system equipped with an Oxford EPR-900 continuous-flow cryostat. The intensity of $g = 4.1$ signal in the Cl^--depleted sample was measured after subtraction by that in the dark-adapted sample. EPR conditions are shown in the figure legends.

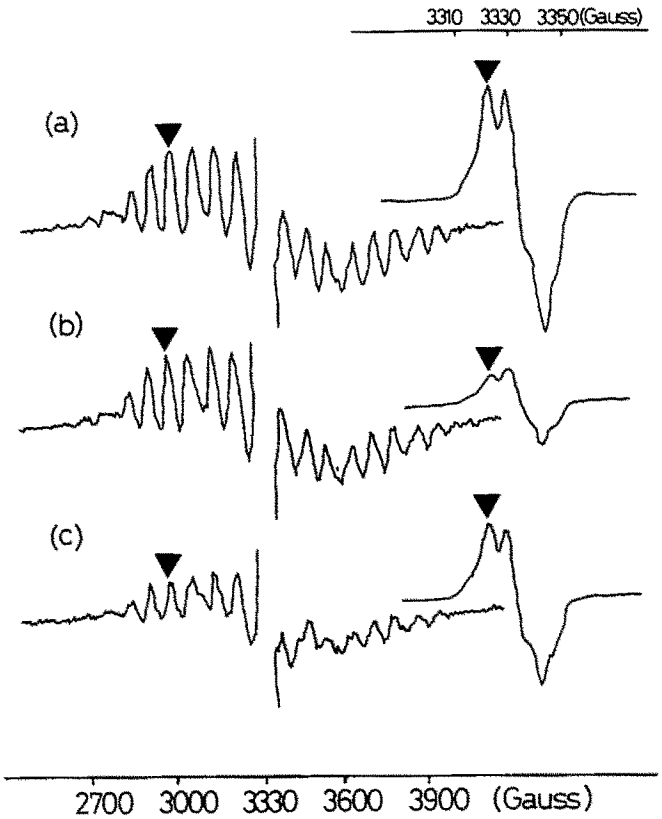

Fig. 1. Comparison of the multiline and Sig. II_s intensities observed at the positions indicated by closed triangles in the illuminated PS II membranes; (a) immediately after illumination at 195 K; (b) after 3 weeks storage at 77 K in the dark; and (c) after dark adaptation at 210 K for 30 min of the same sample as in (b). EPR conditions; microwave frequency 9.37 GHz, power 0.2 mW and 0.2 μW, 100 kHz modulation amplitude 20 G and 5 G for observation of the multiline signal and Sig. II_s, respectively. The field sweep range and gain for Sig. II_s shown in the right upper part of each figure are one-tenth of those for multiline. The temperature was 4.5 K.

Sig. II_s was also measured with a Varian E109 system at 77 K by using a finger-type insertion dewar, or at temperatures from 120 to 220 k by using a nitrogen gas-flow cryostat to observe only its intensity variation during dark storage. The microwave power is fixed at 0.05 mW to avoid saturation in the whole temperature range above 77 K. Cr^{3+}-doped MgO attached on the side-wall of the TE_{102} cavity was used as a reference of intensity calibration over a long period of measurement as in Ref. 9.

Results

In Fig. 1a we show the multiline and the Sig. II_s in the sample immediately after illumination at 195 K. The signals after 3 weeks storage at 77 K are shown in Fig. 1b. The Sig. II_s decayed by storage, while the multiline intensity remained unchanged. The signals shown in Fig. 1c were observed after 30 min dark-adaptation at 210 K of the same sample. The multiline intensity decreased as the intensity of Sig. II_s increased. Sig. II_s recovered most of its original intensity within 40 min,

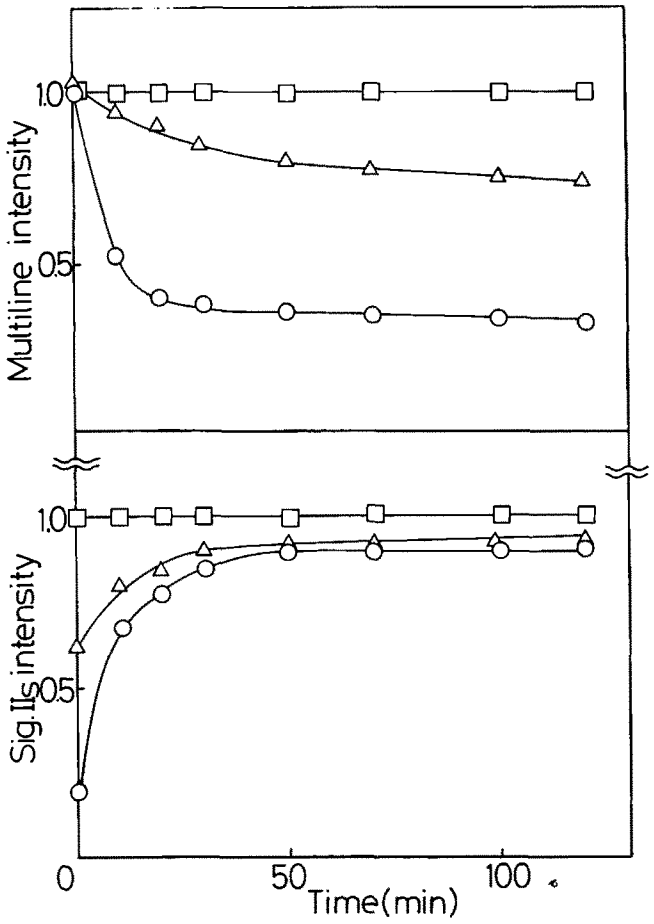

Fig. 2. Effect of the decay ratio of Sig. II_s intensity on the recovery of Sig. II_s and the decay of multiline signal after variable storage time at 210 K; in samples with 0% decay in Sig. II_s intensity immediately after illumination, 40% decay after 3 days storage at 77 K, and 80% decay after 3 weeks storage at 77 K. Assuming $I(t)/I(0) = A + (1 - A)\exp(-t/\tau_d)$, the decay ratios of Sig. II_s were determined after subtraction of A, 10 to 15% of the original intensity. EPR conditions were same as in Fig. 1. □, 0%; △, 4%; ○, 80%.

and its intensity remained same after further adaptation at 210 K as seen from the data with the decay ratio 80% in Fig. 2. The multiline intensity decreased rapidly during first 30 min in a synchronous way with the recovery of Sig. II_s intensity.

The same experiments were performed by using samples which have different decay ratios of Sig. II_s, 80 to 0% prepared by changing storage periods at 77 K. The initial Sig. II_s intensity was determined by taking a correction of 10–15% of the original Sig. II_s intensity immediately after illumination, which remained without decay after long periods of storage of the illuminated sample at 77 K. This part of the Sig. II_s intensity can be considered to originate from the damaged membrane during preparation, and not to contribute to charge transfer in PS II. Using these samples, we observed variation in both Sig. II_s and multiline intensity during dark-adaptation at 210 K. Some of results are shown in Fig. 2. It was evident that the multiline decay must have

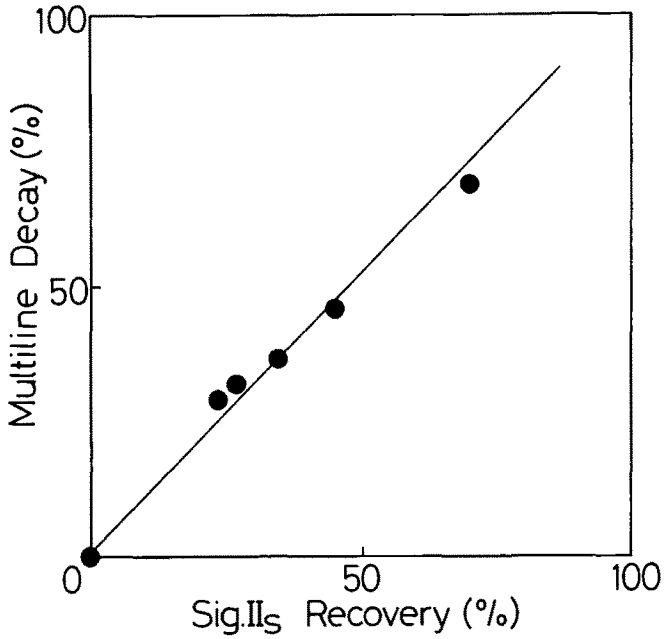

Fig. 3. Correlation of ratios of the Sig. II_s recovery and the multiline decay derived from the data in Fig. 2 and others.

a strong correlation with the recovery of Sig. II_s. In Fig. 3, the decay ratio of multiline intensity and the recovery ratio of Sig. II_s intensity after 2 h dark storage were plotted.

Secondly, the change in the intensity of EPR Sig. II_s in the PS-II was observed at 77, 140 and 180 K during variable storage periods, and the relative intensities are shown in Fig. 4. The intensity at the $g = 2.0117$ position immediately after ilumination at 195 K was taken to be 100%. The characteristic time of decay, τ_d, determined by assuming a single-exponential decay, was about 49.7 h at 77 K, 15.4 h at 140 K, and 9.1 h at 180 K. It decreased as the storage temperature increased in the range 77–180 K.

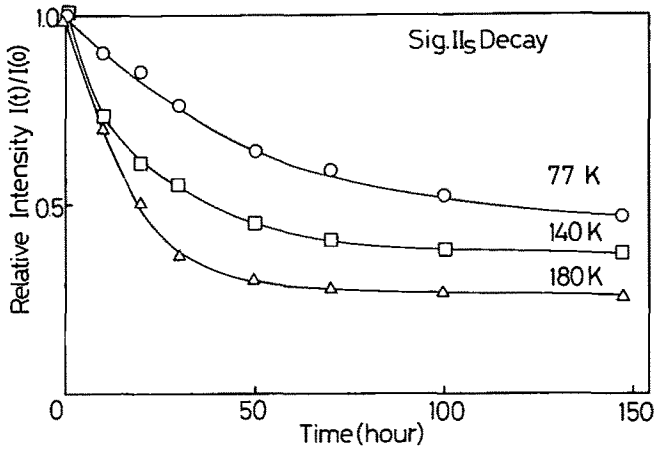

Fig. 4. Change of normalized intensity $I(t)/I(0)$ of the EPR Sig. II_s at the $g = 2.0117$ position in the PS II membranes illuminated at 195 K during variable storage time at 77, 140 and 180 K. $I(0)$ was taken as the intensity immediately after illumination.

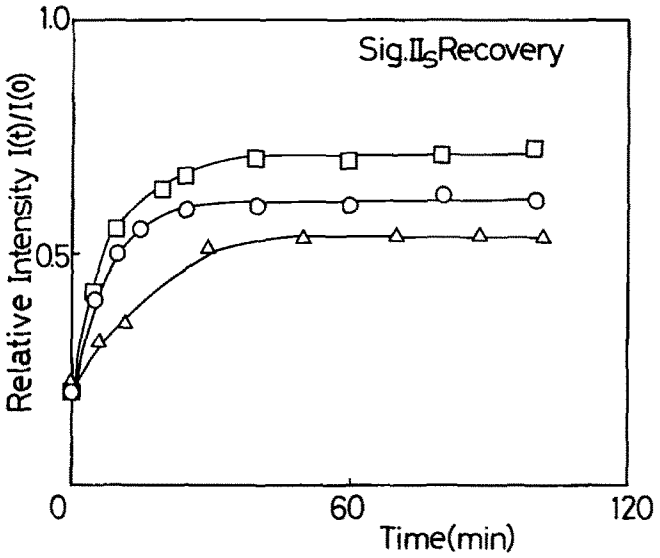

Fig. 5. Change of normalized intensity of $I(t)/I(0)$ of the EPR Sig. II_s observed during variable time of dark-adaptation at 200, 210 and 220 K in the samples storaged at 77 K for 3 weeks after illumination at 195 K. △, 200 K; ○, 210 K; □, 220 K.

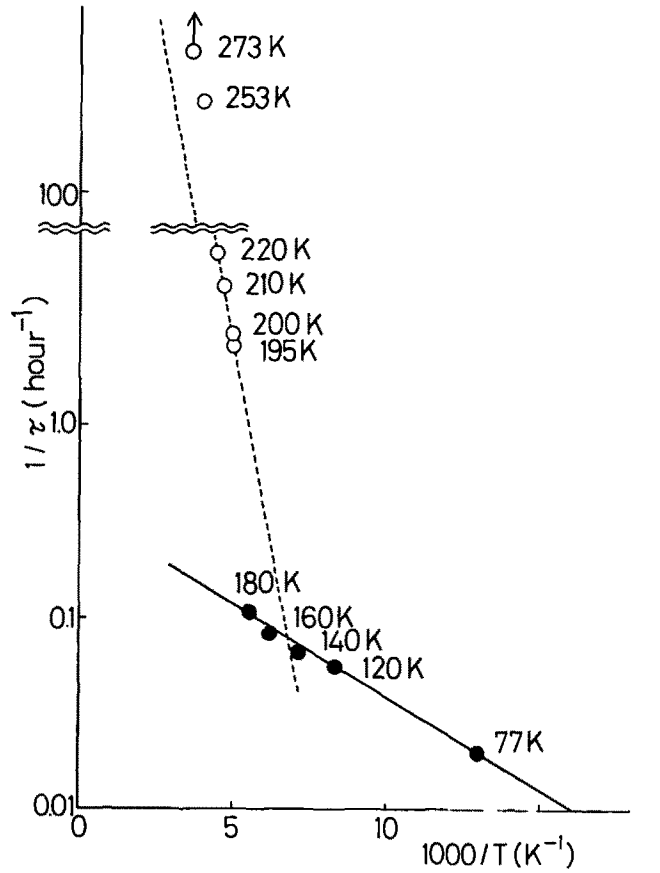

Fig. 6. Decay rates $1/\tau_d$ (●) and recovery rates $1/\tau_r$ (○) of the Sig. II_s intensity derived from the data in Fig. 4, 5 and others at various temperatures. The values for temperatures 253 and 273 K were taken from experiments at 4.5 K, and others were taken from EPR experiments at the indicated temperatures. The full line on experimental values is drawn by Eqn. 1 with the activation energy $U_d = 2.0$ kJ/mol and the broken line with $U_r = 16.6$ kJ/mol. The arrow on the data 273 K indicates that the value could not be measured exactly because the rate was too fast. ○, Sig. II_s recovery; ●, Sig. II_s decay.

On the other hand, EPR intensity in the sample stored at 77 K for 3 weeks after 195 K illumination was measured at 210 K, and recovery in the relative intensity of Sig. II_s was observed in a short time (see Fig. 5) with the characteristic time τ_r of 0.18 h. The recovery of Sig. II_s intensity was obtained at several temperatures between 195 and 220 K, and was found to be much faster than the decay of Sig. II_s. The rates of decay $1/\tau_d$ and recovery $1/\tau_r$ at several temperatures are plotted against $1000/T$ (K^{-1}) in Fig. 6. Activation energies of decay and recovery of the Sig. II_s intensity, which are given by the following equation, were calculated from Fig. 6.

$$1/\tau_{d,r} = A \exp(-U_{d,r}/kT) \quad (1)$$

The calculated activation energy U_d for decay was 2.0 kJ/mol and U_r for recovery was 16.6 kJ/mol. At 253 K, the value of τ_r was 12 s, and at 273 K, it was shorter than 7 s. Accordingly, at elevated temperatures, U_r seemed to be greater.

Corresponding values for the rate of decay in the multiline intensity in the same sample as in Fig. 6 are plotted in Fig. 7. Decay in the multiline intensity immediately after illumination was observed at temperatures above 243 K. The results are shown in Fig. 7 in

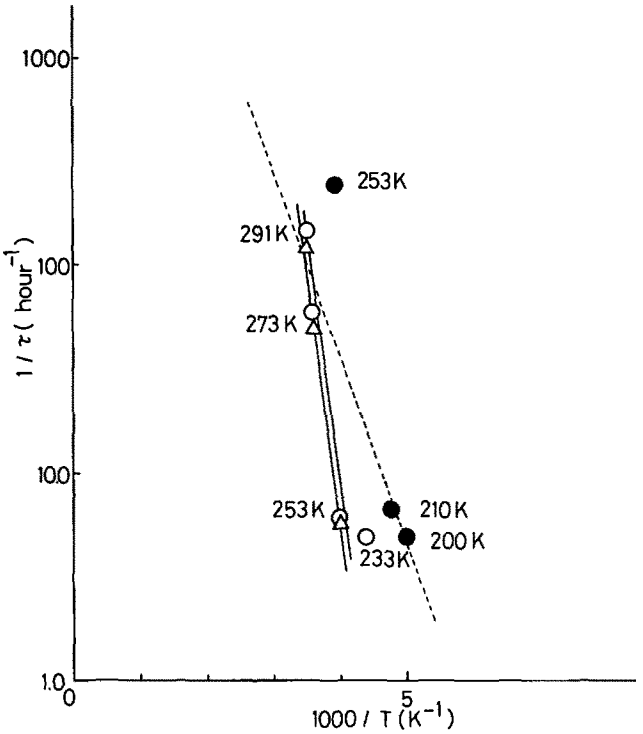

Fig. 7. Rates of multiline decay $1/\tau$ at various temperatures of dark-adaptation after illumination. ●, values for samples stored at 77 K for 3 weeks after illumination. ○, values for samples treated with DCMU and immediately after illumination at 195 K. △, rates in the control sample immediately after illumination. The dotted line was drawn by the activation energy 16.6 kJ/mol and the full line by the activation energy 46.6 kJ/mol.

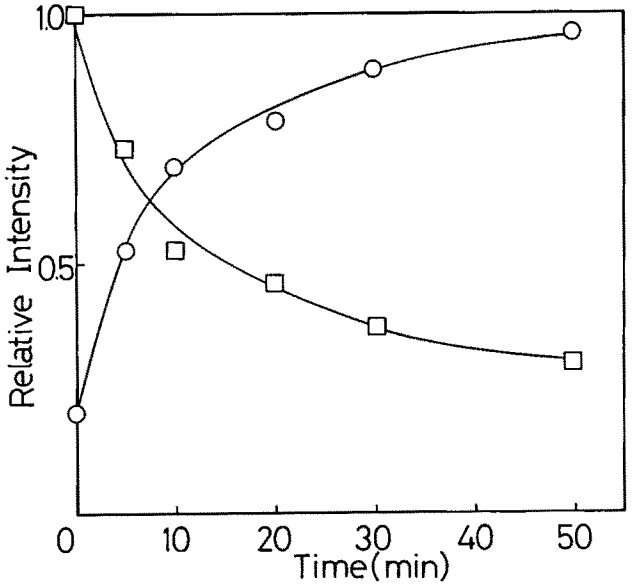

Fig. 8. Decay of the $g = 4.1$ EPR signal in the Cl^--depleted PS-II membranes subtracted by that in the un-illuminated ones and recovery of Sig. II_s with varying time of dark-adaptation at 210 K after 3 weeks storage of the illuminated sample at 77 K. EPR conditions: microwave power 2 mW and others the same as for the multiline. □, $g = 4.1$ signal; ○, Sig. II_s.

comparison with those in samples with Sig. II_s decayed. DCMU accelerated the rate of multiline decay a little. The activation energy, U, was calculated to be 46.6 kJ/mol in both samples with and without DCMU, immediately after illumination.

Thirdly, we studied the decay and the recovery of Sig. II_s in Cl^--depleted and Br^--substituted samples. The characteristic time of decay at 77 K was 51.4 h for the Cl^--depleted and 53.4 h for the Br^--substituted samples. The values of characteristic time for recovery measured at 200 K were 0.27 h for Cl^--depleted and 0.28 h for Br^--substituted samples. We also measured characteristic times in both samples at other temperatures, and got similar values for characteristic times as in the control PS II at the same temperatures (data not shown). We found that the characteristic times of the decay and the recovery of Sig. II_s in Cl^--depleted and Br^--substituted samples were close to those of the control sample, and within the experimental error of about 10%.

In the last experiment, we studied the $g = 4.1$ EPR signal generated by illumination of the Cl^--depleted sample at 195 K. This sample was stored at 77 K for 3 weeks after illumination at 195 K. The intensity of the $g = 4.1$ signal remained almost unchanged. Once the temperature of the sample was elevated to 210 K, the intensity of the $g = 4.1$ signal decreased rapidly, as shown in Fig. 8. The decrease was similar to that in the ordinary PS II with 80% Sig. II_s decay, as shown in Fig. 2. The characteristic time for decay of the $g = 4.1$ signal was approx. 15 min, and the value was close to that for the multiline decay at 210 K.

Discussion

Our present studies are restricted to the recombination path from Q_A to the S-system as is seen in Fig. 1. The process necessarily includes the donor, D, and its oxidized form D^+, and is given by:

$$D^+ + Q_A^- \xrightarrow{\text{below 180 K}} D + Q_A \quad (2)$$

for the decay of Sig. II_s and Q_A, as is shown in Fig. 1b.

$$D + S_2 \xrightarrow{\text{above 200 K}} D^+ + S_1 \quad (3)$$

for the recovery of Sig. II and decay of the multilines is shown in Fig. 1c. The quantitative confirmation of Eqn. 2 has been difficult, because the line-width of the signal due to Q_A^- is broad and other signals due to cytochrome b_{559} overlap [8]. Qualitative confirmation has been done by our previous study and by Nugent et al. [8,9]. On the other hand, the quantitative confirmation of Eqn. 3 was possible at 4.5 K from observation of both the Sig. II_s and the multiline intensity at the same time. The relation between Sig. II_s recovery and multiline decay was plotted in Fig. 3 after correction of Sig. II_s intensity, as mentioned in the previous section. The one-to-one correspondence in the linear relation shown in Fig. 3 indicates that the electron on D transferred directly to the S_2 site in the water-splitting system without any stable intermediate.

The temperature dependence of charge recombination rate measured by Sig. II_s intensity is summarized in Fig. 6. The obtained activation energy, U_d, the recombination process between Q_A^- and D^+, is only 2.0 kJ/mol, showing that the electron on Q_A moved to D^+ slowly among rather low potential barriers. On the other hand, recombination between the S_2 state and D is very fast, and its activation energy, $U_r = 16.6$ kJ/mol between 195 and 220 K, is much higher than that for the process of Eqn. 2.

Furthermore, the activation energy tends to be higher above 253 K, though we could not measure the recovery rate exactly at 273 K. The value at 253 K in Fig. 6 deviates from the extended line from 220 K, indicating that the activation energy is temperature-dependent. Thus, various kinds of molecular motions or different kinds of molecular configurations between D and the manganese complexes may be considered to contribute to the electron transfer with raising temperature. Above 253 K, the contribution from Q_B and quinone pools is also probable because of a redox equilibrium between Q_A and Q_B.

The activation energy for the decay of multiline intensity in Fig. 7 is almost the same as that for Sig. II_s

at temperatures 200 and 220 K, which is consistent with the linear relation shown in Fig. 3. The decay rate in multiline intensity observed in the sample immediately after illumination at 195 K was much slower compared to that in the sample with Sig. II_s decayed. The obtained value of 46.6 kJ/mol for an activation energy has the similar magnitude as that obtained 33.5 kJ/mol by Brudvig et al. [17]. This indicates that some other reductants take the place of D. The most potent reductant other than D is Q_A, and the next one may be Q_B. It is interesting to see that the sample with DCMU, which inhibits electron transfer from Q_A to Q_B, shows a slightly faster decay rate. The charge recombination without DCMU consists of two ways: via Q_A and also via Q_B with a slower decay rate. The order of the rate of recombination $1/\tau$ above 250 K in these redox couples is given by $1/\tau(D) > 1/\tau(Q_A^-) > 1/\tau(Q_B^-)$.

At a low temperature below 250 K where Q_A to Q_B electron transfer is inhibited, and the rate of direct Q_A to S_2 transfer is slowed down, the recombination path is limited to the one path from Q_A to S_2 intermediated by D. Conformational change in proteins around these charge carriers may affect activation energies as suggested by Koike et al. [20] in thermophylic cyanobacterium. The molecular configuration in proteins including D as a residue may be also different at low temperature from that at higher temperatures, resulting in a different pathway and activation energy. As mentioned above, the rate of charge recombination via D is most effective, showing that this radical species is important to recover a redox equilibrium after illumination at physiological temperature.

Decay and recovery rates of Sig. II_s in Br^--substituted and Cl^--depleted samples are almost the same as those discussed in the last section, indicating that the charge recombination path, $Q_A \rightarrow D \rightarrow S_2$ site, was not changed by these ion substitutions below 220 K. This confirms that these ions change the condition of the S_2 state negligibly, although a possibility for modification of the S_2 state, a band shift of few degrees, was found in thermoluminescence of the Cl^--depleted PS II membranes [21]. The deactivation of the S_2 state has been done within some integral proteins and not in the anion-substituted region.

The decay characteristic for the $g = 4.1$ signal in the Cl^--depleted sample shown in Fig. 8 shows a strong correspondence with that for the multiline decay in Fig. 2, indicating that the charge recombination process in both samples are the same in the S_2 state, and that the same redox couples contribute to the process. The modified S_2 state [21] responsible for the $g = 4.1$ signal is also the manganese cluster, though the difference in EPR signal reflects a slightly different exchange coupling in the manganese cluster [16]. The shift of thermoluminescence in the anion-substituted PS II membranes may also be ascribed to a minor conformational change.

In conclusion, the property of Sig. II_s investigated here demonstrates that the radical species, D^+, contributes to the most rapid charge recombination process after illumination. The recombination between D^+ and Q_A^- is thermally activated with its activation energy of 2.0 kJ/mol. The activation energy for recombination between the D and the S_2 state varies with temperature from 16.6 to 46.6 kJ/mol, suggesting the existence of various charge-transfer paths related to various molecular configurations around residue D and the water-splitting system. The anion substitutions have little effect on the charge-recombination process, indicating that the process is governed by conformations of integral proteins, and that the anion-substituted region does not contribute to deactivation of the S_2 state. The $g = 4.1$ signal originates from a manganese cluster similar to that of the ordinary PS II membranes.

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