Interaction of the high-spin Fe atom in the photosystem II reaction center with the quinones Q_A and Q_B in purified oxygen-evolving PS II reaction center complex and in PS II particles

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EPR signals in the high-spin region were studied at 10 K in photosystem II (PS II) particles and in a purified oxygen-evolving PS II reaction center complex under oxidizing conditions. PS II particles showed EPR peaks at $g=8.0$ and 5.6, confirming the recent report by Petrouleas and Diner [(1986) Biochim. Biophys. Acta 849, 264–275]. Addition of 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea (DCMU) or *o*-phenanthroline shifted the peaks to be closer to $g=6.0$ depending on the medium pH. On the other hand, the PS II reaction center complex showed peaks at $g=6.1$ and 7.8, and at $g=6.1$ and 6.4, in the absence and presence of *o*-phenanthroline, respectively. All these peaks were found to be decreased by the illumination at 10 K. These results suggest that the high-spin signals are due to Q_{400}, Fe(III) atom interacting with the PS II primary electron acceptor quinone Q_A as reported and that the Fe atom also interacts with the secondary acceptor quinone Q_B. This interaction seems to induce the highly asymmetric ligand coordination of the Fe atom and to be affected by DCMU and *o*-phenanthroline in a somewhat different manner.

EPR *Photosystem II* *Reaction center* *Plastoquinone* *Fe EPR signal* Q_{400}

1. INTRODUCTION

In a recent study on thylakoid membranes of a *Chlamydomonas* PS I-deficient mutant, Petrouleas and Diner [1] reported new EPR signals in the high-spin Fe region at liquid He temperature. The signals at $g=8$, 6.4 and 5.5 were induced by oxidation with a redox midpoint potential of about 400 mV at pH 7.0 [1]. Addition of DCMU, which inhibits the oxidation of the PS II primary acceptor quinone Q_A^-, by the secondary acceptor quinone (Q_B), eliminated these peaks and increased a peak at $g=6.4$ [1]. The EPR peaks were attributed to the Fe(III) atom associated with Q_A [1,2] (which is now known to correspond to Q_{400} as characterized by Bowes et al. [3] after reinvestigation of the original finding of Ikegami and Katoh [4]). The conclusion was also confirmed by the study of reaction kinetics of Q_A by measuring fluorescence yield, ultraviolet absorption change and Mössbauer spectroscopy [1,2]. Q_{400} is characterized by a pH-dependent redox midpoint potential (400 mV at pH 7.0 and changes -60 mV/pH unit [3]), rapid photoreduction (within 1 μs) and very slow reoxidation rate, especially in the presence of DCMU [3]. These characteristics were reported for the high-spin EPR signals [1].

We extended the study of Petrouleas and Diner

Abbreviations: DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; Mops, 3-(*N*-morpholino)propanesulfonic acid; PS, photosystem; RC, reaction center; Q_A and Q_B, PS II primary and secondary acceptor quinones, respectively

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[1] by measuring EPR signals in the purified oxygen-evolving PS II RC complex of Tang and Satoh [5], which does not have functionally active Q_B [6], and in the PS II particles [7] which have Q_B. Comparison of the EPR data in these two preparations adds new information on the interaction of the Fe atom with Q_A and Q_B to that obtained in the study of the Q_A^-Fe(II) state in PS II [9,10] and in bacterial RC complex [11–15].

2. MATERIALS AND METHODS

PS II particles were derived from spinach leaves according to Kuwabara and Murata [7]. This preparation is estimated to have 2.9 plastoquinone-9 molecules/PS II reaction center (= on the basis of 220 chlorophyll *a* molecules) [8] and shows rapid reoxidation of Q_A^- after the flash excitation (Itoh, S., unpublished). Oxygen-evolving PS II RC complex was prepared from spinach chloroplasts according to Tang and Satoh [5]. The complex contains 6 types of polypeptides (47, 43, 33, 32, 30 and 9 kDa), 3 manganese atoms, 2 plastoquinone-9, 10 carotenoid, 2 pheophytin and about 50 chlorophyll *a* molecules on the basis of one PS II RC [5,6] and shows oxygen-evolving activity almost insensitive to DCMU [6]. Measurement of the amount of oxygen evolved per flash under repeptitive flash excitation according to Kawamura et al. [16] with phenyl-*p*-benzoquinone as an electron acceptor indicated that more than one third of the RCs are fully active in oxygen evolution. This preparation shows a very slow dark oxidation of Q_A^- (taking more than 10 min) in the absence of added electron acceptor (Itoh, S. and Tang, X.-S., unpublished).

EPR spectra were recorded on a Bruker ER-200 X-band spectrometer using an oxford Instruments ESR-900 continuous flow cryostat in the Center of Analytical Instruments of National Institute for the Basic Biology as reported [17]. EPR samples in 3-mm i.d. quartz tubes were dark-adapted for about 1 h at 0°C followed by another 30 min dark incubation with inhibitors before being frozen to 77 K in liquid nitrogen. Samples were illuminated in a cryostat by light from a 150 W high-pressure mercury lamp through a heat cut filter (Hoya HA-50) by means of a glass fiber light guide.

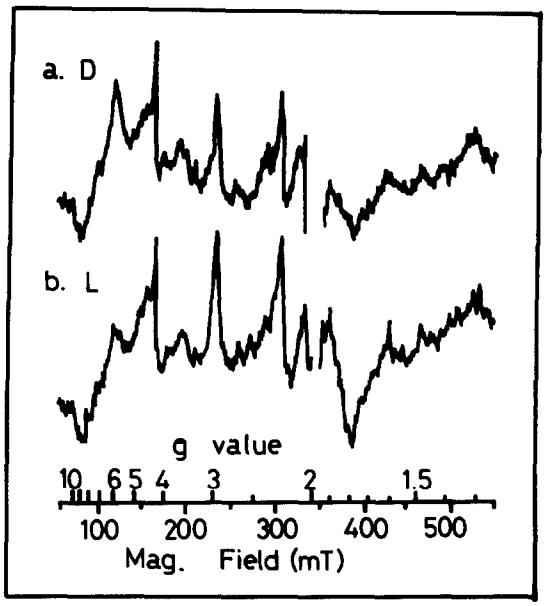


Fig.1. EPR spectrum of oxygen-evolving PS II RC complex at 10 K. (a) Dark-adapted sample; (b) after illumination at 100 K for 2 min. PS II RC complex (0.23 mg chlorophylls/ml) was suspended in a medium containing 100 mM NaCl, 50 mM Mes-NaOH buffer (pH 6.0) and 50 mM $CaCl_2$. Modulation amplitude, 4 mT, microwave frequency, 9.76 GHz; Microwave power, 100 mW. Gain 6×10^4. Signals were averaged four times.

3. RESULTS

3.1. *High-spin Fe EPR signal in PS II RC complex*

Fig.1 shows the wide range EPR spectrum of the PS II RC complex measured at 10 K under various conditions. In the sample frozen in the dark after long dark adaptation, several peaks were observed as shown elsewhere [6]. Peaks at $g=2.95$ and 2.24 are attributed to g_z and g_y bands of oxidized cytochrome *b*-559 (low-potential form [17,18]). The peak positions were the same as those in $CaCl_2$-washed oxygen-evolving PS II particles but were somewhat different from those in Tris-washed (Mn-depleted) ones [17]. In the $g=2.0$ region, signal II (slow) was also observed (not shown). The peak at $g=4.3$ seems to represent free iron. Apart from these signals, there existed a peak at $g=6.1$ in the high-spin Fe region after long dark adaptation at 0°C even in the absence of ferricyanide. Illumination of the sample at 10 K (see

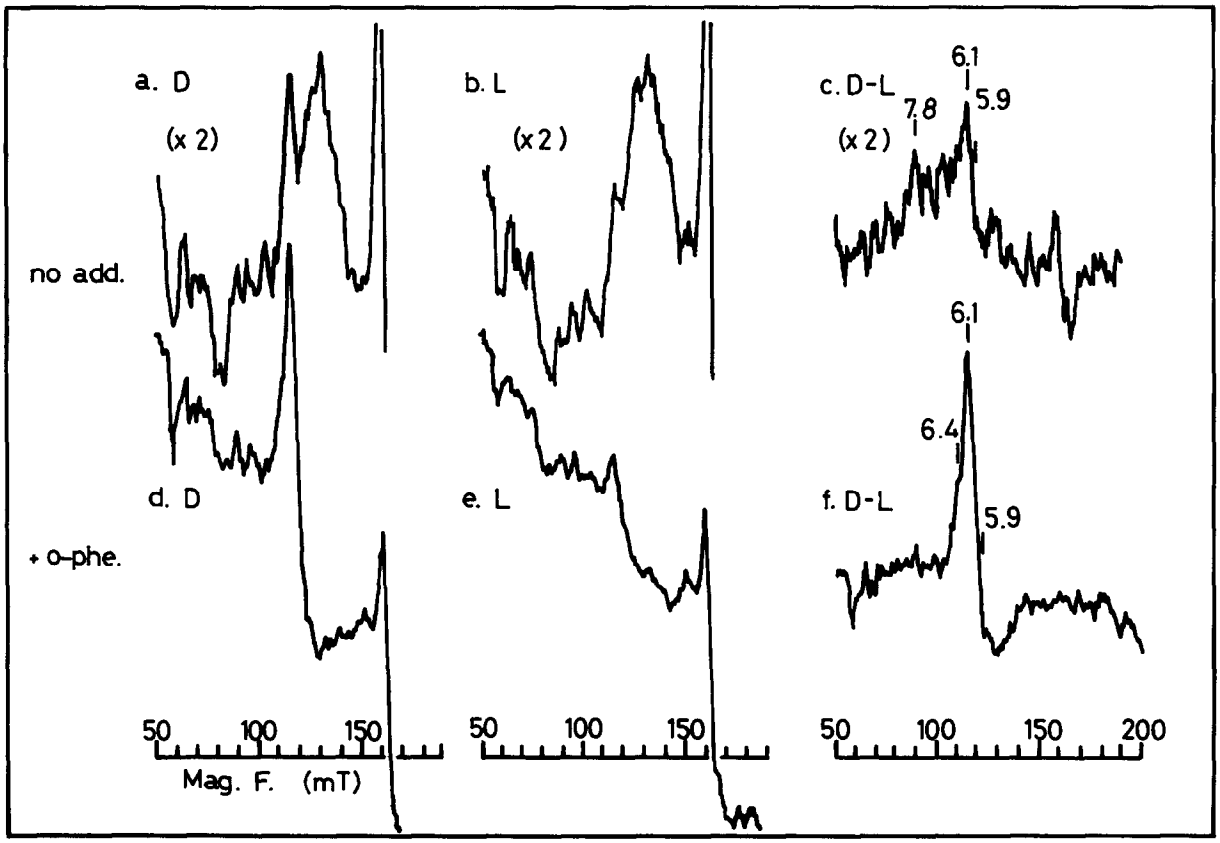


Fig.2. Effects of illumination at 10 K and *o*-phenanthroline on the high-spin EPR spectrum in PS II RC complex. (a) Dark adapted at 0°C with 2 mM ferricyanide; (b) after illumination at 10 K for 2 min; (c) a minus b; (d) dark adapted at 0°C with 2 mM ferricyanide and then dark incubated with *o*-phenanthroline (5 mM final concentration) for another 30 min before being frozen in liquid N_2; (e) after illumination of sample in d for 2 min at 10 K; (f) d minus e. Other experimental conditions were similar to those in fig.1 except that microwave power was decreased to 10 mW.

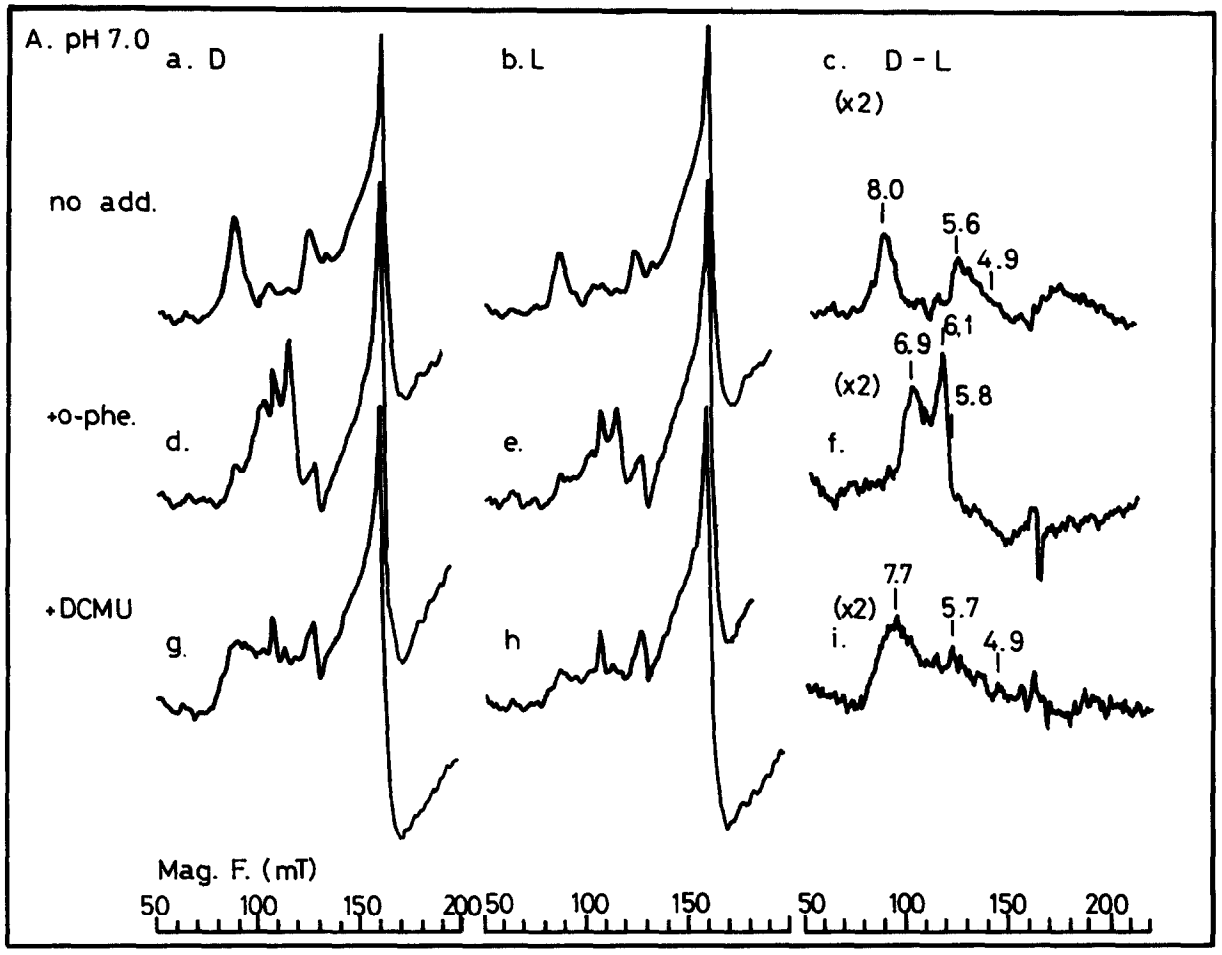
fig.2) partially decreased this peak and induced a large free radical type 10–11 G width large signal at $g=2.0$ region (Signal II_{fr} not shown). The latter is probably due to Car^+ [19,20]. After illumination at 100 K (fig.1b) the $g=6.1$ peak was more significantly depressed and the broad trough centered at $g=1.8$ which is attributed to the $Q_A^-Fe(II)$ complex [9,10] became prominent (fig.1, trace b). After illumination at 200 K, the $g=6.1$ peak was almost completely depressed and the manganese hyperfine signal of the S_2 state of the oxygen-evolving center [21] was also induced in the $g=1.5$–3.0 region, as reported in [6] (not shown). The peaks at $g=4.3$ and those of cytochome *b*-559 (mainly of low-potential form and, therefore, fully oxidized in the dark [6]) were not greatly affected by these illuminations.

In the presence of ferricyanide, the $g=6.1$ signal appeared after shorter dark incubation (less than 30 min) (fig.2, trace a). This signal was partially depressed by illumination at 10 K (trace B) and almost completely eliminated after incubation with ascorbate (not shown). The dark minus illuminated (at 10 K) difference spectrum (trace c) shows a major peak at $g=6.1$ (zero crossing point at $g=5.9$) with a broad smaller peak at about $g=7.8$. Addition of *o*-phenanthroline gave a sharper and more intense peak at $g=6.1$ (zero crossing point at $g=5.9$) with a smaller peak at $g=6.4$ (trace d). The signal is a typical high-spin Fe(III) type having an axial symmetry with a slight rhombicity [22]. Illumination at 10 K eliminated

almost 90% of these two peaks and yielded a difference spectrum with sharp peaks (traces e,f). The results suggest that these signals originate from an Fe(III) atom, which can be photoreduced even at 10 K and has a redox midpoint potential of 300–400 mV at pH 6.0. *o*-Phenanthroline probably increases the symmetry of the ligand coordination around the Fe(III) atom. However, the peak positions of the EPR signals observed in figs 1 and 2 are somewhat different from those attributed to the Fe(III) interacting with Q_A in *Chlamydomonas* membranes previously [1] or in the PS II particles as will be shown in section 3.2.

3.2. *Effects of inhibitors and pH on the high-spin Fe signals in the PS II particles*

EPR signals in the high-spin region were also studied in the PS II particles. The particles have both the Q_A and Q_B quinones. This situation is in contrast to that in the PS II RC complex which lacks the Q_B quinone. Dark preincubation of PS II particles with ferricyanide at 0°C induced two large peaks at $g=8.0$ and 5.6 with a zero crossing point at $g=4.9$ (the peak sometimes appeared to split into two peaks) and a very small derivative type signal at about $g=6.4$ at pH 7.0 (fig.3A, trace a). The peak positions were almost the same as reported for membranes of a *Chlamydomonas* mutant [1], although the intensity of the $g=6.4$ peak is much lower than that reported [1]. The illumination at 10 K depressed these peaks (trace b). The signal can be studied more accurately in the dark-adapted minus 10 K-illuminated difference spectrum (trace c). On addition of *o*-phenanthroline, the peaks were sharper and shifted to $g=6.9$ and 6.1 (zero crossing point at $g=5.8$). On addition of



DCMU, the peaks became broader and the dark-minus-illuminated difference spectrum yielded peaks at $g=7.7$ and 5.7 (zero crossing point at $g=4.9$), which positions are intermediate between those in the presence and absence of *o*-phenanthroline (fig.3, trace i). The signal shape in the presence of DCMU is somewhat different from that reported in [1], probably due to the difference in experimental conditions such as medium pH.

The positions of the peaks determined in the absence of inhibitors did not change when the medium pH was elevated to pH 8.7 (fig.3B), although the intensities of the peaks became higher. Addition of *o*-phenanthroline induced a large peak at $g=6.1$ with a zero crossing point at $g=5.9$ and a shoulder at $g=6.4$, with concomitant diappearance of the two peaks at $g=8.0$ and 5.6. On addition of DCMU, a similar signal but with a lower intensity was induced by ferricyanide with a peak at $g=6.1$ (with a zero crossing point at $g=5.8$) and a shoulder at $g=6.3$ (traces g–i). A peak at $g=7.9$ seen in trace i may represent the

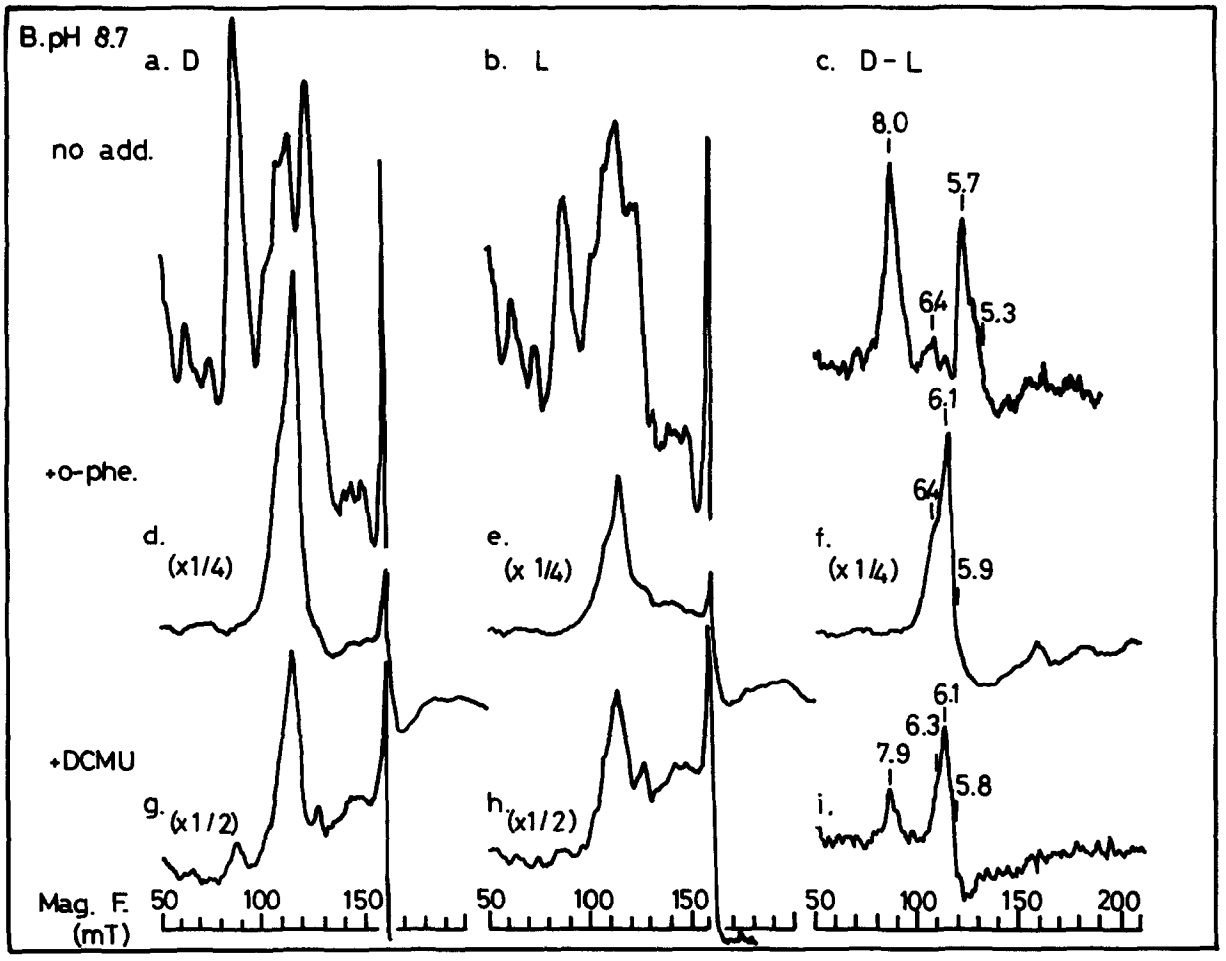


Fig.3. Effects of *o*-phenanthroline and DCMU on the high-spin EPR signals at 10 K in PSII particles at pH 7.0 (A) and at pH 8.7 (B). (a) Dark-adapted; (b) after illumination of sample in a for 2 min at 10 K; (c) a - b; (d) dark adapted with 5 mM *o*-phenanthroline; (e) after illumination of sample in d for 10 min at 10 K; (f) d minus e; (g) dark adapted with 10 μM DCMU; (h) after illumination of sample in g for 2 min at 10 K; (i) g minus h. Reaction mixture contained 2 mM ferricyanide, 50 mM Tris-HCl buffer pH 7.0 (A) or pH 8.7 (B), 5 mM $MgCl_2$, 100 mM NaCl and PS II particles equivalent to 2.6 mg chlorophyll/ml. Modulation amplitude 4 mT. Microwave frequency 9.60 GHz. Microwave power 10 mW. Gain 6×10^4.

heterogeneity of the sensitivity of the RCs to DCMU. The relative signal intensities were 1 ($g=8.0$ peak in the absence of inhibitors):4.4 ($g=6.1$ in the presence of *o*-phenanthroline):1.6 ($g=6.1$ in the presence of DCMU) at pH 8.7. The difference in effectiveness between DCMU and *o*-phenenthroline seems to agree with the conclusion by Wraight [23] obtained by the study of Q_{400} kinetics. It is clear that in the presence of *o*-phenanthroline or DCMU at least two peaks are observed at pH 7.0, which become closer to each other at pH 8.7 indicating a more axial symmetry. The fact that the signal intensity increased significantly at the higher pH, especially in the presence of *o*-phenanthroline, seems to indicate pH-dependent changes of the ligand coordination around the Fe atom.

Signals in the $g=2.0$ region were also studied in the RC complex and in PS II particles (not shown). In each case illumination at 10 K induced the 10–11 G width signal (Signal II_f), which seems to be due to a carotenoid$^+$ [19,20] closely associated with P680$^+$, to a similar extent.

4. DISCUSSION

The characteristics of EPR signals in the PS II particles may be summarized as follows. Ferricyanide oxidation induces two major EPR peaks at $g=8.0$ and 5.6, which are affected by DCMU and depressed by illumination at 10 K as reported [1]. The signal at $g=6.4$ reported in [1] was very small and was not characterized well. The signal intensities of the major $g=8.0$ and 5.6 peaks depended on the redox conditions of the medium in a parallel manner, showing an apparent midpoint potential of about 400 mV as reported [1], a value which is almost the same as that of Q_{400} [1,3,4].

Addition of *o*-phenanthroline or DCMU increased the signal intensity, especially at alkaline pH. *o*-phenanthroline was more effective than DCMU. The inhibitors also shift the peak positions from $g=8.0$ and 5.4 to the more axial positions close to $g=6.0$. The effect was larger with *o*-phenanthroline than with DCMU, and was more pronounced at higher pH. In the purified PS II RC complex similar signals were observed even in the absence of inhibitors and ferricyanide, but in this preparation the peaks were found at $g=6.1$ and 7.8. Also in this case $g=$ o-phenanthroline increased the signal intensity at $g=6.1$ and shifted the smaller peak from $g=7.8$ to $g=6.4$. 10 K illumination depressed the intensities of all these signals. The high-spin signal does not parallel the Q_A^-Fe(II) state signal at $g=1.8$, since the latter was, under the present experimental conditions, noticeable only after the illumination. These results confirm the conclusion previously obtained [1] that the high-spin signals originate from the Fe(III) atom interacting with Q_A, i.e. Q_{400}. DCMU or *o*-phenanthroline are known to inhibit electron transfer between Q_A^- and Q_B probably by displacing Q_B from its binding site [24,25]. It may therefore be concluded that the displacement of Q_B molecule from its binding site in the vicinity of the Fe atom by the inhibitors or by the extraction during the intensive detergent treatment [5,6] results in the shift of g values to the more axial position close to $g=6.0$, especially at alkaline pH. This may be due to some changes in the ligand coordination of the Fe atom caused by the modification of the Q_B binding site. The binding site of *o*-phenanthroline seems to be different from that of DCMU and still exist in the RC complex which retains the QB binding protein but lacks Q_B. The photochemistry in the ferricyanide-oxidized PS II RC seems to proceed in the following way at 10 K:

Car P680 Q_A-Fe(III)-$Q_B \rightarrow$ Car$^+$ P680 Q_A-Fe(II)-Q_B

in which Car$^+$ and Fe(III) are EPR active.

EPR signal in the presence of inhibitors can be assumed to originate from a high-spin iron with $g_x=6.1$ and $g_y=5.9$ (zero crossing point) in analogy with usual high-spin nonheme Fe(III) signal [22]. Then the effects of inhibitors may be understood to increase the symmetry of the centers by decreasing their rhombicity ($g_x=7.8\rightarrow6.1$, $g_y=$ less than $4.6\rightarrow5.9$). On the other hand, in the RC complex, the Fe(III) center has a symmetry of ligands similar to that seen in inhibitor-treated PS II particles even without the inhibitors. It may be concluded that binding of Q_B molecule to its binding site in the Q_B binding 33-kDa protein, directly or indirectly, produces the highly asymmetric structure in the coordination of the Fe(III) atom and induces the $g=8.0$ peak. This seems to be in line with the structure of the homologous Fe(II) atom in the bacterial RC, in which the Fe atom is estimated to be octahedrally coordinated by ligands of N atoms of other amino acids between Q_A and Q_B ubiquinone molecules from the

study of EPR [11-13] or EXAFS [14,15] spectroscopy and by X-ray crystallography [26]. A similar structure can also be estimated in PS II from the EPR measurement of the $Q_A^-Fe(II)$ state at $g = 1.8$ region [9,10]. However, there is as yet no report of high-spin signals of the Fe atom in the bacterial RC. The ligands of the PS II Fe atom may have pK values between 7 and 9 and may be responsible for the pH dependence of the high-spin signals. The binding sites of *o*-phenanthroline and DCMU can be concluded to be a little different from each other.

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