*Biochimica et Biophysica Acta*, 1017 (1990) 204–208
Elsevier

BBABIO 43228

# Location of $D^+$ and distribution of surface charges in Photosystem II

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(Received 10 November 1989)
(Revised manuscript received 13 March 1990)

Key words: Photosystem II; EPR; Oxygen evolution; Tyrosine radical; (Spinach chloroplast)

**Intramembrane position of $D^+$, the unusually stable tyrosine radical in Photosystem II (PS II), has been estimated by analyzing the effect of an electron-spin relaxing agent dysprosium (Dy) on the microwave power saturation of the EPR signal of $D^+$. It is shown that an estimation with an electrostatically neutral complex of Dy with hydroxyEDTA (HEDTA) gives more reliable information on the structural organization of PS II than that with a charged $Dy^{3+}$ ion or Dy-$EDTA^-$ complex. Distance from $D^+$ to the outer surface of PS II was estimated at 36 Å from the data with Dy-HEDTA. The distance to the inner surface was estimated at 28 Å with the three (33, 24 and 18 kDa) peripheral polypeptides associated or at 20 Å without these polypeptides. By comparison of the effect of Dy-HEDTA with those of $Dy^{3+}$ and Dy-$EDTA^-$, the inner surface newly exposed after the removal of the 24 and 18 kDa polypeptides is shown to be negatively charged and after the removal of the 33 kDa polypeptide to be positively charged.**

## Introduction

A tyrosine residue of a reaction center polypeptide of PS II, termed Z, participates in electron transfer between the reaction center chlorophyll (P-680) and the manganese center of the oxygen-evolving complex [1–3]. Another tyrosine in PS II is unusually stable in its radical form in darkness and is designated as D. D can react with $P\text{-}680^+$ and also with the manganese center [4]. However, the redox turnover of D is considerably slow and its physiological role is still unclear. The oxidized forms of Z and D give spectrally identical but kinetically different EPR signals called Signal $II_{vf}$ (or $II_f$) and Signal $II_s$, respectively. These tyrosine residues are assumed to be situated symmetrically, with P-680 interposed, in the PS II reaction center complex [2,3,5].

We have studied the effects of an electron-spin relaxing agent dysprosium (Dy) on the microwave power saturation of $D^+$, to investigate structural organization and charge distribution on the inner surface of PS II [6–8]. We used two forms of the relaxing agent, i.e., positively charged $Dy^{3+}$ ion and negatively charged Dy-$EDTA^-$ complex, and concluded that $D^+$ was situated close to the inner side of thylakoid membrane. The surface near $D^+$ seemed to be negatively charged and to be covered by the peripheral 33, 24 and 18 kDa polypeptides of the oxygen-evolving complex. Following our reports, Innes and Brudvig [9] gave a general expression which correlates the effect of the relaxing agent with the distance between a signal species and the membrane surface. They suggested a model for the location of $D^+$ in PS II based on the analysis with Dy-$EDTA^-$. In their model, D is located near the middle of the membrane, which is unexpected from the PS-II structure deduced from the amino acid sequence of the reaction center polypeptide on the basis of the structural homology between PS II and bacterial reaction centers [5,10]. In this work, we have studied the effects of an electrostatically neutral complex, Dy-HEDTA, on the EPR saturation of $D^+$. The effect of Dy-HEDTA is compared with those of $Dy^{3+}$ and Dy-$EDTA^-$, and the neutral complex is revealed to be the most suitable tool for the structural study of PS II. The results also give information on charge distribution of the inner surface of PS II. We have revised the method

Abbreviations: Chl, chlorophyll; Dy-HEDTA, Dy-hydroxyEDTA complex; EPR, electron paramagnetic resonance; Mes, 4-morpholineethanesulfonic acid; PS II, Photosystem II; P-680, the reaction center chlorophyll of PS II; Signal $II_s$, $II_f$ and $II_{vf}$, EPR Signal $II_{slow}$, $II_{fast}$ and $II_{very\ fast}$, respectively; Dy, dysprosium.

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of the distance calculation, and propose a model for the spatial organization of PS II on the analysis with Dy-HEDTA. A preliminary account of this work has been presented in Ref. 11.

## Materials and Methods

Thylakoid membranes and PS-II particles were prepared from market spinach leaves according to Ref. 12 with minor modifications described in Ref. 13. Selective removal of the peripheral polypeptides (33, 24 and 18 kDa) from the PS-II particles was carried out as previously described [7]. The samples were finally suspended in 0.4 M sucrose, 50 mM Mes-NaOH (pH 6.5), 10 mM NaCl and 30 % (w/v) ethylene glycol at 3 mg Chl/ml.

The PS-II particles were mixed with Dy-HEDTA (or La-HEDTA, which was used as a control) and incubated at 4° C for 15 min in darkness before they were frozen in liquid nitrogen. Dy-HEDTA and La-HEDTA were obtained by mixing $DyCl_3$ and $LaCl_3$ with a 10% excess of HEDTA, respectively, followed by adjustment of pH to 6.5. The suspension was transferred into a quartz EPR tube and frozen in liquid nitrogen in darkness. The first derivative EPR spectra were recorded at 20 K using a Bruker ER-200 X-band spectrometer fitted with an Oxford Instruments ESR-900 liquid helium cryostat.

The power saturation of Signal $II_s$ was analyzed by plotting $S/\sqrt{P}$ against $P$ both on logarithmic scales, where $S$ and $P$ are the amplitude of the lowest-field peak and microwave power in mW, respectively [14], as shown in Fig. 1. The microwave power at which $S/\sqrt{P}$ is one-half of its unsaturated value is taken for a saturation parameter $P'_{1/2}$ [8].

## Results and Discussion

The microwave power saturation of $D^+$ was relieved by addition of Dy-HEDTA in thylakoid membranes without significant changes in the spectral lineshape (Fig. 1). The effect increased with concentration of the relaxing agent. No effect of La-HEDTA which was added for control was observed on the EPR signal under the experimental conditions used.

Fig. 2 shows the effect of Dy-HEDTA on the saturation of $D^+$ in three types of the PS-II particles; untreated particles which retained the peripheral 33, 24 and 18 kDa polypeptides [15], NaCl-washed particles which preserved only the 33 kDa polypeptide [16,17] and the $CaCl_2$-washed particles which had no peripheral polypeptides [18]. The saturation parameter $P'_{1/2}$ showed an approximately linear dependence on the concentration of Dy-HEDTA in the thylakoid membranes and the PS-II preparations (Fig. 3), as previously reported for $Dy^{3+}$ and Dy-EDTA$^-$ [7,9]. The change in $P'_{1/2}$ ($\Delta P'_{1/2}$) by addition of Dy-HEDTA was 0.067 mW/mM for the thylakoid membranes. $\Delta P'_{1/2}$, which measures accessibility of the relaxing agent, increased

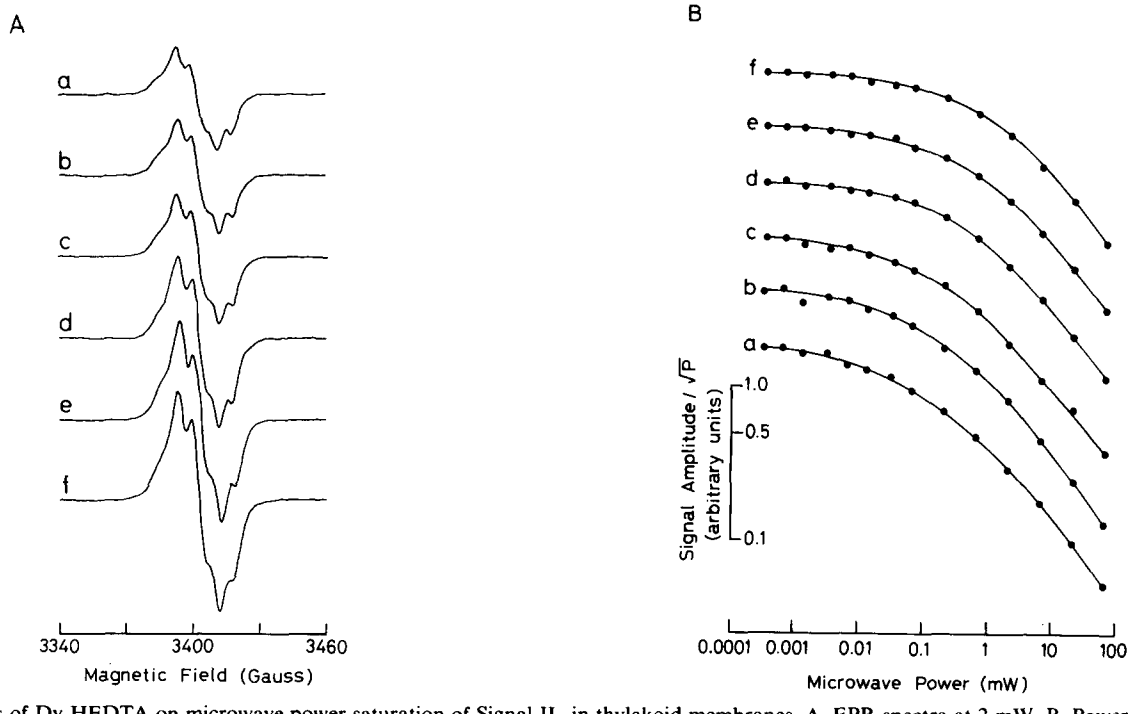


Fig. 1. Effects of Dy-HEDTA on microwave power saturation of Signal $II_s$ in thylakoid membranes. A, EPR spectra at 2 mW. B, Power saturation profiles. The power saturation of Signal $II_s$ was analyzed by plotting $S/\sqrt{P}$ against $P$ both on logarithmic scales, where $S$ and $P$ are amplitude of the lowest-field peak and microwave power in mW, respectively. La-HEDTA (2 mM) was added for the control (a). Dy-HEDTA was added at concentration of 2 mM (b), 4 mM (c), 7 mM (d), 10 mM (e) and 15 mM (f). EPR conditions: temperature, 20 K; microwave frequency, 9.62 GHz; modulation amplitude, 4 G; scanning rate, 2 G/s; time constant, 160 ms.

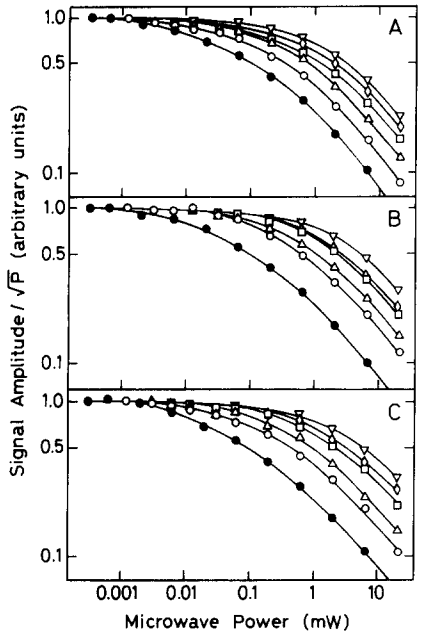


Fig. 2. Effects of Dy-HEDTA on the power saturation profile of Signal $II_s$ in PS-II particles. A, Untreated particles; B, NaCl-washed particles; C, $CaCl_2$-washed particles. The concentration of Dy-HEDTA was 0 (●), 2 (○), 4 (△), 7 (□), 10 (◇) and 15 (▽) mM. EPR conditions are the same as in Fig. 1.

by fragmentation of the thylakoid membranes into the PS-II particles and further by the selective removal of the peripheral polypeptides.

In Ref. 7, we reported that the effects of $Dy^{3+}$ on the saturation of Signal $II_s$ were significantly larger than those of Dy-$EDTA^-$ and that the removal of the peripheral polypeptides increased the accessibility of these relaxing agents to $D^+$. The changes in $P'_{1/2}$ by Dy-HEDTA are compared with those by $Dy^{3+}$ and Dy-$EDTA^-$ in Table I. The effectiveness of Dy-HEDTA was intermediate between those of $Dy^{3+}$ and Dy-

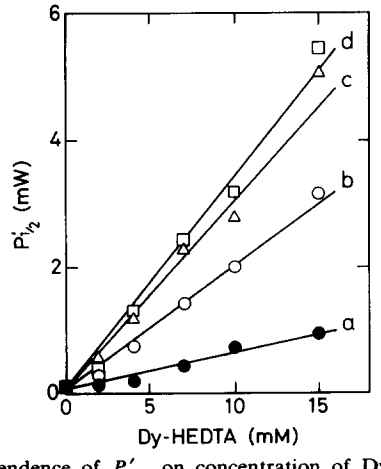


Fig. 3. Dependence of $P'_{1/2}$ on concentration of Dy-HEDTA. The microwave power at which $S/\sqrt{P}$ is one-half of its unsaturated value is taken for the saturation parameter $P'_{1/2}$. a, thylakoid membranes (●); b, untreated PS-PP particles (○); c, NaCl-washed particles (△); d, $CaCl_2$-washed particles (□).

TABLE I

*Changes in $P'_{1/2}$ by the relaxing agents*

| PS-II particles | $\Delta P'_{1/2}$ (mW/mM) | | |
|---|---|---|---|
| | $DyCl_3$ [a] | Dy-EDTA [a] | Dy-HEDTA |
| Untreated | 2.5 | 0.06 | 0.20 |
| NaCl-washed | 5.1 | 0.06 | 0.33 |
| $CaCl_2$-washed | 5.3 | 0.20 | 0.37 |

[a] Data from Ref. 7.

$EDTA^-$ in our experiments. This shows that the accessibility of $Dy^{3+}$ and Dy-$EDTA^-$ is affected by the distribution of surface charges as well as the structural organization. The effect of neutral Dy-HEDTA, on the other hand, seems to be determined mainly by the structural organization.

We calculated distances from $D^+$ to the membrane surfaces from the data with Dy-HEDTA (Table II), assuming $r^{-3}$ dependence of $\Delta P'_{1/2}$. The dependence is derived from a model in which the Dy-HEDTA complexes randomly distribute in the aqueous phase surrounding a planar membrane [9]. Previously, we applied the $r^{-6}$ dependence of $\Delta P'_{1/2}$ to the distance calculation

TABLE II

*Distances from $D^+$ to inner and outer surfaces of thylakoid membrane*

From the assumption of random dispersion of Dy in solution surrounding planar membrane in which $D^+$ is buried [9], the relationship between $\Delta P'_{1/2}$ and distance from $D^+$ to the membrane surface is given by:

$$\Delta P'_{1/2}\ (\mathrm{mW/mM}) = C\left(r_1^{-3} + r_2^{-3}\right) \qquad (1)$$

where $r_1$ and $r_2$ are distances from $D^+$ to the outer and inner surfaces of PS II, respectively, and $C$ is constant. The membrane thickness $(r_1 + r_2)$ for the $CaCl_2$-washed PS-II particles, is assumed to be 56 Å (data from electron microscopy of the purified PS-II complex [19]). In case of the thylakoid vesicle in which Dy can not access from the inner side, $r_2^{-3}$ will be negligible. Thus, the following relationship is obtained from Eqn. 1:

$$\left(\Delta P'_{1/2}\right)_{\mathrm{PS\,II}} / \left(\Delta P'_{1/2}\right)_{\mathrm{tkd}} = \left((r_1+5)^{-3} + (r_2+5)^{-3}\right) / (r_1+5)^{-3} \qquad (2)$$

where $(\Delta P'_{1/2})_{\mathrm{PS\,II}}$ and $(\Delta P'_{1/2})_{\mathrm{tkd}}$ are $\Delta P'_{1/2}$ values for the PS-II particles and thylakoid membranes, respectively, and 5 Å for the size of Dy-HEDTA complex. By the use of Eqn. 2, the distances from $D^+$ to the outer and inner surfaces in each preparation of the PS-II particles are obtained without knowing the $C$ value.

| Sample | Distance (Å) |
|---|---|
| From $D^+$ to outer surface thylakoid membranes | 36.1 |
| From $D^+$ to inner surface | |
| untreated PS II particles | 27.7 |
| NaCl-washed particles | 21.2 |
| $CaCl_2$-washed particles | 19.9 |

[6] according to the method of Blum and Ohnishi [14], in which it was assumed that only the relaxing agent binding at a single site had the significant contribution to the change of $P'_{1/2}$. The $r^{-3}$ dependence used here, however, was shown to give more reliable estimation of the distance [9]. Here we used the thickness of 56 Å for the membrane with no peripheral polypeptides as a calibration value, which was obtained from the electron-microscopical study of the PS-II complex purified from cyanobacteria [19]. The use of the PS-II membrane itself for calibrating the Dy effect seems to give more accurate estimation of the distance compared with the use of other systems such as myoglobin nitroxide or the bacterial reaction center as in Ref. 9. In the calculation, we also assumed an additional 5 Å from Dy to the membrane surface for the complex size of Dy-HEDTA.

$D^+$ is located at 36.1 Å from the inner surface of the untreated PS-II particles (Table II). The removal of the 24 and 18 kDa polypeptides by the NaCl washing decreased the distance by about 7 Å, whereas the further removal of the 33 kDa polypeptide by the $CaCl_2$ washing decreased the distance by only 1 Å. This indicates that the 24 and 18 kDa polypeptides bind closer to $D^+$ than the 33 kDa polypeptide. The 33 kDa polypeptide, which is involved in binding manganese onto PS II [22–24], may be associated with the membrane surface near Z rather than D. This agrees with the recent EPR study [25] which suggested a rather long distance between $D^+$ and the manganese center (30–40 Å).

The distance allotted for these peripheral polypeptides (approx. 8 Å) is much smaller than the radii (21, 19 and 17 Å for 33, 24 and 18 kDa proteins, respectively) calculated from their molecular masses and the partial specific volume of protein (0.72 $cm^3 \cdot g^{-1}$) for spherical molecules. This may be due to the flat shapes of these peripheral polypeptides [19] and also due to the assumption that the inner surface of the PS-II membrane is planar even when the membrane binds the peripheral polypeptides.

Innes and Brudvig [9] suggested a structural model for the location of $D^+$ in PS II based on the data with Dy-$EDTA^-$. In this model, $D^+$ is located near the middle of the membrane (26 Å from the outer surface; 41 and 27 Å from the inner surface with and without the peripheral polypeptides, respectively). They reported that the neutral Dy-HEDTA complex caused the same effect as the negatively charged Dy-EDTA complex in the untreated PS-II particles and concluded that the accessibility of Dy-$EDTA^-$ would not be affected by the surface charges of the membrane. This contradicts our results (Table I) and the reason for the inconsistency is uncertain. The difference between the distances estimated with Dy-$EDTA^-$ [19] and with Dy-HEDTA (Table II), however, may partially be due to the fact that the inner surface of PS II is more negative than the outer surface. They used the Tris-treated particles, which had no peripheral polypeptides nor manganese, for estimation of the distance from the inner surface without the peripheral polypeptides. The accessibility of Dy-$EDTA^-$ ($Dy^{3+}$) in the $CaCl_2$-washed particles was larger (smaller) than that in the Tris-treated particles, possibly due to positive charges of manganese associated with the $CaCl_2$-washed particles [8]. The effect of Dy-HEDTA, on the other hand, had little difference between the $CaCl_2$-washed and Tris-treated preparations (not shown). The study on the kinetics of $Z^+$ in Tris-treated PS II with charged electron donors [21], also showed high density of negative charges on the inner surface. The data in Table I clearly indicates that the effects of $Dy^{3+}$ and Dy-$EDTA^-$ were affected by the charge distribution on PS II, and the use of these charged agents will not provide proper information on the structural organization.

The location of $D^+$ by Innes and Brudvig [9] seems to be unexpected from the PS-II structure deduced from the X-ray structure of the bacterial reaction center and the sequence homology of the D2 protein in PS II with the M subunit of the bacterial reaction center [2,3,5,10]. Our estimation with Dy-HEDTA (Table II) is compatible with the proposed structure of PS II.

Tables I and II also give information on surface charge distribution of PS II. The NaCl washing increased the accessibility of Dy-HEDTA, indicating removal of the spatial hindrance from the membrane. The NaCl washing also increased the accessibility of $Dy^{3+}$ but caused little effect on that of Dy-$EDTA^-$. This suggests that the negatively charged surface on the PS-II complex is exposed by the removal of the 24 and 18 kDa polypeptides. On the other hand, the $CaCl_2$ washing increased the accessibility of Dy-$EDTA^-$ more significantly than that of $Dy^{3+}$ and Dy-HEDTA, indicating exposure of positive charges by the removal of the33 kDa polypeptide. A part of the exposed positive charges may be due to manganese of the oxygen-evolving complex because the effects of $Dy^{3+}$ and Dy-$EDTA^-$ increased and decreased, respectively, by the removal of manganese [6,8].

When charge distribution on the membrane surface is changed, but the effect of the removal of spatial hindrance is small, as in case of the removal of manganese, a complementary effect of a charged relaxing agent for the other relaxer with the opposite charges should be observed. However, in the case of the removal of the peripheral polypeptides, the effects of the charged relaxers will be determined by the sum of two factors. One is change of the surface charges as seen in the removal of manganese. The other is change of the distance from $D^+$ to the surface exposed to the relaxing agent. Because of the contribution of the second factor, simple complementary effects of the relaxers with op-

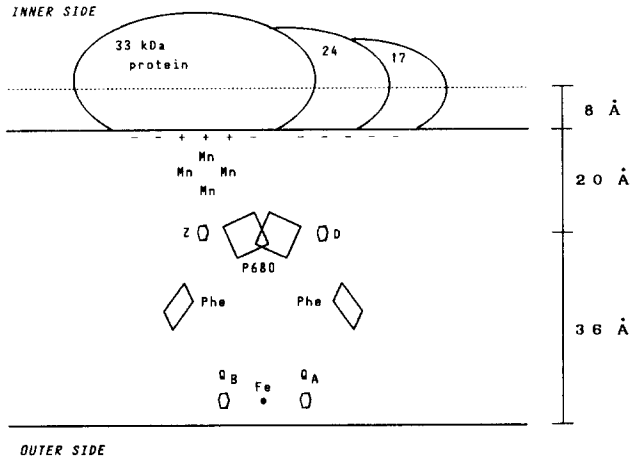


Fig. 4. Structural model of PS II. The dashed line shows the distance allotted for the peripheral polypeptides by the analysis of the data with Dy-HEDTA (see text). The reaction center chlorophyll of PS II (P-680), pheophytin (Phe), quinones for the electron acceptors ($Q_A$ and $Q_B$) are located on the analogy with the reaction center of purple nonsulfur bacteria [5].

posite charges are not expected. The results in Table I, however, indicate that $Dy^{3+}$ and Dy-EDTA$^-$ give the complementary effects if one compares their effects with that of neutral Dy-HEDTA.

Based on the present work and also on the homology of the reaction center polypeptides between PS II and purple nonsulfur bacteria [5,10], we propose a model for the location of $D^+$ and the peripheral polypeptides in PS II in Fig. 4. This model also shows the spatial relationship between the cofactors of the reaction center and those of the oxygen evolving complex, and is consistent with the recent biochemical [26,27] and biophysical [25,28] studies on the organization of PS II.

## Acknowledgements

We appreciate Dr. Yoshihiko Fujita (National Institute for Basic Biology, NIBB) for helpful discussion and Ms. Masayo Iwaki (NIBB) for technical assistance. Y.I. also thanks Dr. Tetsutaro Iizuka (RIKEN) for kind advice on the EPR experiment. This work was supported in part by Grants-in-Aid from the Ministry of Education, Science and Culture, Japan.

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