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# Assignment of histidine residues in D1 protein as possible ligands for functional manganese in photosynthetic water-oxidizing complex

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**By use of a chemical modifier, the ligand amino-acid residue(s) for manganese ligation in the photosynthetic water-oxidation center was investigated. The following was found. (1) Treatment with diethyl pyrocarbonate (DEPC), a histidine modifier, caused a loss of photoactivation capability of $NH_2OH$-treated PS II membranes (devoid of Mn). (2) DEPC-induced loss of photoactivation capability showed pH dependence with a $pK_a$ of 6.9 and was reversed by subsequent treatment with $NH_2OH$, both indicative of specific involvement of histidine residue in the modification. (3) DEPC modification was protected by the presence of Mn photoligated in the center, and DEPC-modified centers drastically lost the Mn binding affinity accompanied by a decrease in rate of generation of the unstable intermediate state involved in the multi-quantum process of photoactivation. (4) [$^{14}$C]DEPC treatment radiolabelled several PS II proteins, but the labelling intensities of D1 and a 60 kDa band due to D1/D2 heterodimer were specifically suppressed among others by the presence of photoligated Mn. Based on this, it was inferred that D1 protein contains a ligand histidine residue(s) specifically interacting with Mn functional in water oxidation.**

## Introduction

In algae and higher plants, oxidation of Mn proceeds following the unique Joliot-Kok's oxygen clock driven by photoreaction in PS II to oxidize water to oxygen [1]. The four Mn atoms functioning in accumulation of oxidizing equivalents are clustered on PS II intrinsic proteins (47 kDa, 43 kDa, D1 and D2 as a pair of dimers [2,3]) or a tetramer [3], in which a Mn atom is approx. 2.7 Å away from the nearest Mn atom and approx. 3.3 Å away from the other two [4]. It is also considered that a part of functional Mn may be bound to the extrinsic proteins of 33 [5] or 23 kDa [6] under certain conditions. According to the recent concensus, Mn(III) in the low S state is likely to be the principal valence state, which increases to Mn(IV) as the S state is advanced [7] (however, see also Ref. 8).

Precise identification of the proteins providing principal ligation of Mn still remains to be done. Particularly, elucidation of amino-acid residues involved in photoactivation of the apo-complex is an essential facet for understanding the mechanism of water oxidation. Recently, Klein and his colleagues [4] demonstrated by X-ray absorption spectroscopy of PS II membranes that either an N or an O atom is ligated with Mn. The candidate for the N-donor atom is highly likely to be a histidine residue, a typical ligand for the metal ion in biological macromolecules at physiological pH [9]. On the other hand, as for the amino-acid residues for the O-donor atom, tyrosine and carboxyl-containing amino acids may be candidates.

Recently, we have succeeded in photoligating $Mn^{2+}$ to inactivated PS II membranes which lack both Mn and PS II extrinsic proteins [10,11]. By use of this experimental system, the present study aims at identification of amino-acid residues involved in photoligation of Mn during photoactivation of inactivated water-oxidizing complex. For this purpose, $NH_2OH$-treated

Abbreviations: PS, Photosystem; TMF2, oxygen-evolving Triton-X-100-prepared Photosystem II membrane fragment(s); DCIP, 2,6-dichlorophenolindophenol; Mes, 4-morpholineethanesulfonic acid; DPC, 1,5-diphenylcarbazide; DEPC, diethyl pyrocarbonate; LHCP, light-harvesting chlorophyll protein.

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PS II membranes were treated with DEPC, a reagent highly reactive with histidine residues [12], and the effects of the treatment on photoactivation were investigated. It was suggested that the histidine residues in D1 protein are involved in photoligation of $Mn^{2+}$ to the apo-complex.

## Materials and Methods

TMF2 were obtained from 7–8-day-old wheat seedlings as described in Ref. 13. $NH_2OH$-treated TMF2 ($NH_2OH$-TMF2) was obtained by the procedure in Ref. 11. The $O_2$-evolving reaction center was prepared as described in Ref. 14. These preparations were used either directly or after storage at $-80°C$.

Chemical modification of PS II membranes by DEPC was carried out at 20 °C in buffer A (50 mM Mes-NaOH (pH 6.5)/20 mM NaCl/0.4 M sucrose) containing PS II membranes equivalent to 100 μg Chl/ml and 500 μM DEPC, unless otherwise noted. The reaction was stopped at a given time by addition of 20 mM histidine in buffer A, washed twice in buffer A, and then resuspended in buffer A. The DEPC-treated membranes were subjected to photoactivation as described in Refs. 10 and 11; the membranes (250 μg Chl/ml) were incubated with 1 mM $MnCl_2$/50 mM $CaCl_2$/100 μM DCIP in buffer A at 20 °C for 45 min under weak light illumination (approx. 25 $\mu E/m^2$ per s). After photoactivation, $O_2$-evolving activity was measured to determine the loss in photoactivation capability induced by DEPC treatment.

Radiolabelling of PS II membranes with [$^{14}$C]DEPC was done by treating the PS II membranes (250 μg Chl/ml) with 1.25 mM [$^{14}$C]DEPC (Hungarian Academy of Science; 9 mCi/mmol) in darkness or under weak illumination (approx. 25 $\mu E/m^2$ per s).

$O_2$ evolution was determined polarographically as described in Ref. 13. DCIP photoreduction was measured spectrophotometrically at 600 nm on the split mode with a Shimadzu spectrophotometer (UV-300). Mn was determined with a Shimadzu flameless atomic absorption spectrometer (AA-640-13, GFA-3) after one wash of the sample with 2 mM EDTA, as described in Ref. 13, provided no sample digestion took place before the measurement.

SDS-polyacrylamide gel electrophoresis analyses were carried out using a 5% stacking and a 12.5% separating polyacrylamide gel containing 5.5 M urea [15]. Gels were stained with Coomassie brilliant blue R-250, dried, and then subjected to fluorography. Western blotting was done, according to the standard methods [16], using a Bio-Rad immunoblot kit with a nitrocellulose membrane and alkaline phosphatase-conjugated goat anti-rabbit IgG. Rabbit antisera against CP47 apoprotein, CP43 apoprotein, and D1 and D2 proteins were raised by using respective proteins from spinach as antigens [15].

## Results

*Effects of DEPC on $O_2$ evolution and photoactivation*

Fig. 1 depicts the effect of DEPC treatment on $O_2$ evolution and photoactivation capability. $O_2$ evolution by TMF2 was slightly (less than 20%) decreased by a prolonged DEPC treatment (500 μM, 60 min), indicating that DEPC does not much affect the intact Mn cluster in TMF2. In contrast, when $NH_2OH$-TMF2 was exposed to DEPC under the same conditions, the photoactivation capability of the preparation was severely affected: the regeneration of $O_2$ activity was diminished with a half-time of approx. 15 min in parallel with a decrease in light-driven incorporation of Mn. The rate of DEPC-induced inactivation followed apparent first-order kinetics over a wide range of DEPC concentration (data not shown), exhibiting a relatively high rate constant of 92.6 $M^{-1} \cdot min^{-1}$ at pH 6.5. It is inferred from these results that the site(s) for chemical modification by DEPC is/are extensively exposed in the water-oxidizing complex lacking Mn and extrinsic proteins, but not in the intact complex capable of $O_2$ evolution. It is also noted that there is no possibility that histidine modification gives rise to nonspecific, EDTA-washable binding of $Mn^{2+}$ to membranes in darkness (closed triangles in Fig. 1).

Table I shows the effect of DEPC on donor activities of $NH_2OH$-TMF2. DCIP photoreduction supported by DPC or $MnCl_2$ was gradually inhibited during exposure

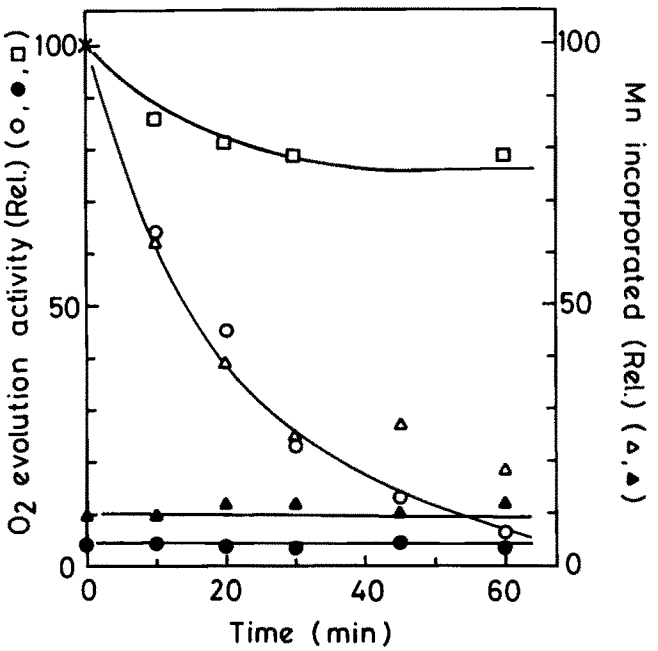


Fig. 1. Time-courses for inactivation of TMF2 and $NH_2OH$-TMF2 by DEPC treatment. TMF2 or $NH_2OH$-TMF2 was incubated with 500 μM DEPC at pH 6.5, 20 °C. □ indicate $O_2$ evolution by TMF2. Circles and triangles represent $O_2$ evolution and Mn incorporation by $NH_2OH$-TMF2, respectively. Open and closed symbols come from photoactivated and non-photoactivated $NH_2OH$-TMF2 membranes, respectively.

TABLE I

*Effects of DEPC treatment on photoactivation and DCIP photoreduction with DPC or $MnCl_2$ as electron donor*

The assay medium for DCIP photoreduction contained DEPC-treated $NH_2OH$-TMF2 equivalent to 5 μg Chl/ml, 30 μM DCIP, 200 μM DPC or 50 μM $MnCl_2$ in buffer A. Photoactivation was carried out as described in Materials and Methods. Electron-transport activities are shown in μmol DCIP/mg Chl per h, and in percentages (in parentheses) as related to those of non-treated samples.

| | DCIP photoreduction | | Photoactivation |
|---|---|---|---|
| DEPC treatment | DPC → DCIP | $MnCl_2$ → DCIP | |
| None | 320 (100) | 59.2 (100) | 203 (100) |
| 15 min | 294 (92) | 55.2 (93) | 128 (63) |
| 60 min | 214 (67) | 36.8 (62) | 34 (17) |

to DEPC, whereas the photoactivation capability was lost far more rapidly. The inactivation of DCIP photoreduction after DEPC treatment for 15 and 60 min was 5–10% and 30–35%, respectively, whereas that of photoactivation were 40% and 80–90%, respectively. These results suggest that DEPC modification of PS II membranes does not much affect the capability of $Mn^{2+}$ photooxidation, but does affect the capability of binding/ligating $Mn^{2+}$. We note that this does not contradict our previous view that photooxidation of $Mn^{2+}$ to $Mn^{3+}$ precedes the ligation of Mn to the apo-complex during photoactivation [11].

DEPC treatment was also found to affect the regeneration by photoactivation of thermoluminescence capability. The glow peak due to $S_2Q_B^-$ charge recombination after a single flash [17] decreased in exact proportion to the loss in $O_2$-evolution activity.

*Assignment of histidine as the modified amino-acid residue*

DEPC specifically reacts with histidine residues in proteins at slightly acidic pH values, while other nucleophilic groups (cysteine, tyrosine and lysine residues) are also modified in neutral or weakly alkaline media [12]. In order to assign the DEPC-modified residue(s) giving rise to the loss in capability of Mn photoligation, the following analyses were carried out: (i) treatment of the modified membranes with $NH_2OH$, which is known to specifically reverse histidine and tyrosine modification [18]; (ii) examining the effect of pH on the inactivation rate, which is known to depend on the dissociation of imidazole proton in histidine residues [19].

$NH_2OH$-TMF2 was treated with DEPC for a given time, followed by incubation with 0.2 M $NH_2OH$ at 4°C at pH 6.5, and then subjected to photoactivation. As shown in Fig. 2, DEPC-induced inactivation of $O_2$ evolution was largely restored by incubation with $NH_2OH$. When 3 h incubation with $NH_2OH$ was employed, the photoactivation yield was recovered to approx. 90, 95 and 60% of the control for membranes exposed to DEPC for 10, 20 and 60 min, respectively. Since $NH_2OH$ is established to remove the ethoxyformyl group from modified histidine or tyrosine residues, but does not cleave the more stable ethoxyformyl–sulfhydryl and *N*-ethoxyformyl–lysyl bonds [12], histidine and/or tyrosine are assigned as the modified residue responsible for the loss in Mn photoligation capability.

Fig. 3 shows the pH dependence of the rate of DEPC-induced inactivation of photoactivation capability. The rate constants were determined by plotting the loss in capability of photoactivation as a function of incubation time with DEPC. The rate constants were small below pH 6.0, but steeply increased with increase in pH, and reached a plateau above pH 7.4. This pH dependence exhibiting a faster modification at alkaline pH values strongly suggests the involvement of histidine, but not tyrosine, residue in the inactivation, since unprotonated histidine is known to react with DEPC much faster than protonated histidine [20]. The apparent rate constant for inactivation, $k_{2(obs)}$, was determined at varying pH values from the pseudo first-order rate constant for the loss of photoactivation capability, and plotted as a function of $[H^+]$ in the inset of Fig. 3. The modification reaction is theoretically a second-order reaction as proposed by Consineau and Meighen [21]. However, if we assume that the rate of loss in photoactivation capability is equal to that of the total number of histidines, and that unprotonated

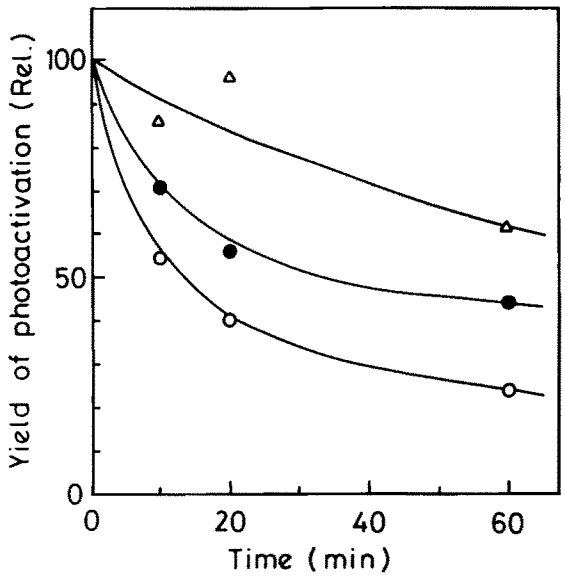


Fig. 2. Reversibility by $NH_2OH$ treatment of DEPC-induced inactivation of photoactivation capability of $NH_2OH$-TMF2. $NH_2OH$-TMF2 was treated with DEPC for indicated periods. After two washes, the treated membranes were incubated with 0.2 M $NH_2OH$ at pH 6.5, 4°C for 0 (○), 1.5 (●) and 3 h (△), and then subjected to photoactivation. The $O_2$-evolution activity regenerated was plotted against the time of DEPC treatment.

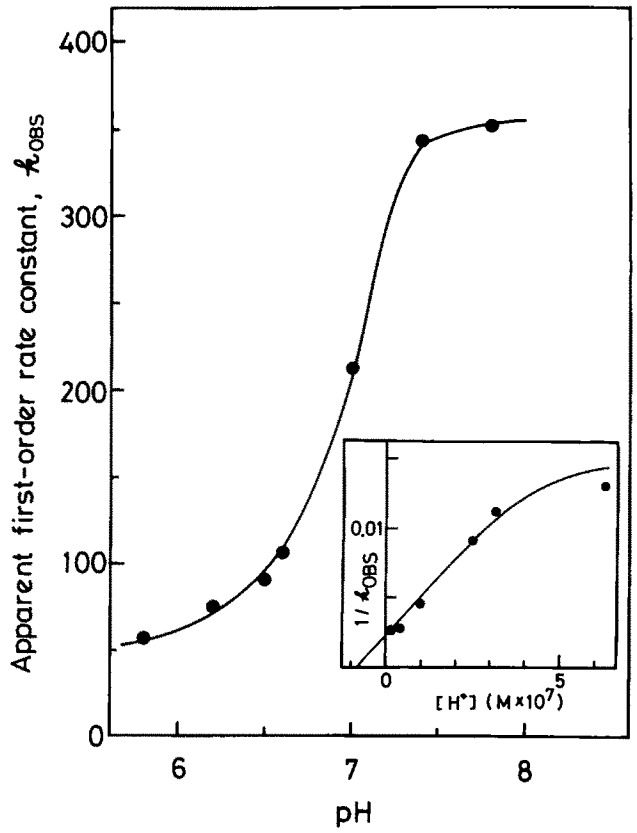


Fig. 3. pH dependence of DEPC-induced inactivation of photoactivation capability in $NH_2OH$-TMF2. $NH_2OH$-TMF2 was treated with 500 $\mu$M DEPC at various pH values for a given time, and then photoactivated at a constant pH of 6.5. Sodium phosphate buffer was used for varying the pH of reaction media. The rate constants were also plotted in the inset as a function of proton concentration according to the method proposed by Cousineau and Meighen [21].

histidines are the only species reactive with DEPC, $k_{2(obs)}$ will be expressed by the following equation:

$$1/k_{2(obs)} = 1/k_2 + [H^+]/k_2 K_a$$

where $k_2$ is the second-order rate constant for formation of covalent bond by unprotonated protein, and $K_a$ is the apparent dissociation constant of protonated form of histidine. From the intercept and slope, $pK_a$ and $k_2$ were calculated to be 6.9 and 435 $M^{-1} \cdot min^{-1}$, respectively. The $pK_a$ value thus obtained coincides with those values reported for modification of histidine residues in various enzymes [19,22]. These results confirm that the loss of capability of photoligation of $Mn^{2+}$ is due to ethoxyformyl formation in histidine residues of PS II intrinsic protein(s) by the action of DEPC.

*$Mn^{2+}$-dependent suppression of DEPC modification*

To examine whether or not the modified histidine residues responsible for the loss of photoactivation capability are located at/near the ligation/binding site of $Mn^{2+}$, the effects of $Mn^{2+}$ and light were investigated (Table II). $NH_2OH$-TMF2 was incubated with DEPC

TABLE II

*Effects of $Mn^{2+}$ and light on DEPC-induced inactivation of photoactivation capability*

$NH_2OH$-TMF2 (1–4) was treated with DEPC in the presence and absence of $Mn^{2+}$ for 60 min in darkness or under weak light preceded by 5 min preillumination, and then photoactivated after two washes. In (5), $NH_2OH$-TMF2 was photoactivated before treatment with DEPC under the same conditions as in (2). $O_2$ evolution ($\mu$mol $O_2$/mg Chl per h) regenerated by photoactivation was directly determined polarographically.

| DEPC treatment | $O_2$ evolution regenerated by photoactivation (%) |
|---|---|
| (1) None | 189 (100) |
| (2) 60 min, dark | 28 (15) |
| (3) 60 min,$Mn^{2+}$, dark | 27 (14) |
| (4) 60 min, $Mn^{2+}$, light (5 min preillumination) | 87 (46) |
| (5) 60 min, photoactivated | 149 (79) |

for 60 min in the presence or absence of $Mn^{2+}$ and illumination. The loss of photoactivation capability due to 60 min incubation was substantially suppressed when the $NH_2OH$-TMF2 was illuminated in the presence of 1 mM $Mn^{2+}$ (Table II, line 4). This suppression required light, showing no effect when $Mn^{2+}$ was employed in darkness (line 3). These results are interpreted as indicating that the Mn-induced protection of histidine residues against DEPC is not contributed by the apocomplex, which binds $Mn^{2+}$ in darkness, but specifically by the potentially active complex which photoligates $Mn^{2+}$ via photoactivation.

*Modulation of Mn binding affinity by DEPC modification*

Hsu et al. [23] reported that submicromolar $Mn^{2+}$ exogenously given to Tris-treated PS II membranes inhibits DPC photooxidation competitively with DPC, depending on pH and anion species as observed for the S-state transition reaction [24]. They suggested that the high-affinity binding site for the exogenous $Mn^{2+}$ is identical with the site for endogenous Mn in the Mn-complex. By use of this phenomenon, although there remained some ambiguities as to its mechanism, we attempted to examine whether or not DEPC modification modulates the Mn-binding affinity (Fig. 4). DPC-supported DCIP photoreduction by DEPC-treated $NH_2OH$-TMF2 was measured in the presence of varied concentrations of exogenous $Mn^{2+}$. In agreement with Ref. 22, we observed that exogenous $Mn^{2+}$ at very low concentrations suppresses the electron donation to PS II by DPC. However, the extent of suppression (at least 60%) was not as high as in Ref. 23, probably due to the following: (1) additional DCIP photoreduction (approx. 20 $\mu$mol DCIP/mg Chl per h) donated by exogenous $Mn^{2+}$, and (2) microenvironmental changes in the high-affinity Mn-binding site induced by $NH_2OH$ treatment.

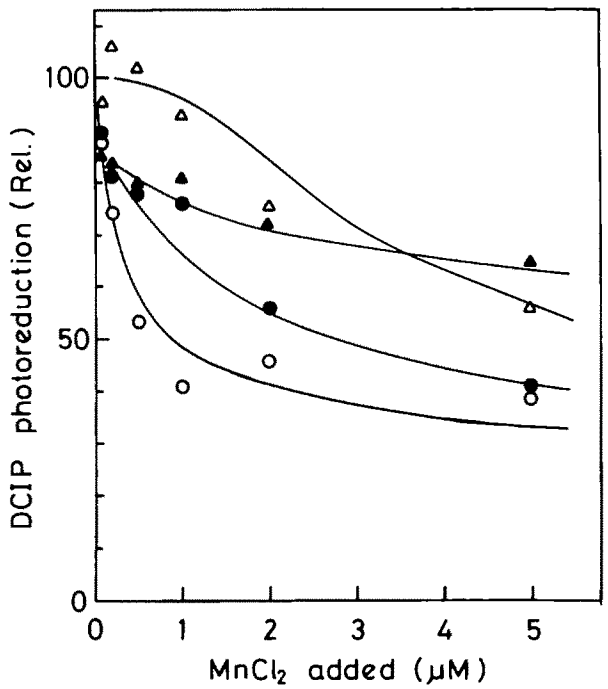


Fig. 4. Exogenous $Mn^{2+}$-induced suppression of DCIP photoreduction by DEPC-treated $NH_2OH$-TMF2 with DPC as the donor. $NH_2OH$-TMF2 was treated with 500 μM DEPC for varying periods: ○, 0 min; ●, 10 min; ▲, 20 min; △, 60 min. The assay medium contained DEPC-treated $NH_2OH$-TMF2 (5 μg Chl/ml), 30 μM DCIP, 200 μM DPC and a given concentration of $MnCl_2$ in buffer A.

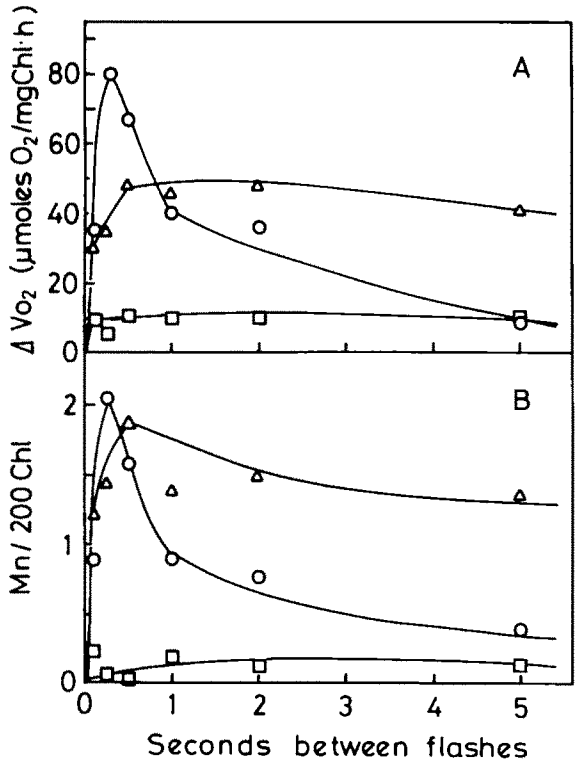


Fig. 5. Flash-interval-dependence of photoactivation yield as expressed by regeneration of $O_2$ evolution (A) and by Mn incorporation (B) in DEPC-treated $NH_2OH$-TMF2. DEPC-treated membranes were illuminated with 720 saturating short flashes (under 5 μs) in the photoactivation buffer (see Materials and Methods), and $O_2$ evolution and the abundance of incorporated Mn were determined after two washes. $NH_2OH$-TMF2 was treated with 500 μM DEPC for 0 min (○), 10 min (△) and 60 min (□).

The $Mn^{2+}$ concentration inducing half-inhibition was approx. 0.25 μM, which is in agreement with the value reported for Tris-treated PS II membranes [23]. With increase in extent of DEPC modification, the $Mn^{2+}$ concentration required for half-inhibition increased, and the activity by the 60-min-treated membranes was almost completely resistant to submicromolar $Mn^{2+}$. This indicates that ethoxyformyl formation in histidine residues drastically decreases the Mn-binding affinity of the Mn binding/ligating sites in PS II membranes.

*Modulation of kinetics of $Mn^{2+}$ photoligation by DEPC modification*

Fig. 5 shows the effect of DEPC modification on the flash interval dependence of photoactivation efficiency, in which DEPC-treated $NH_2OH$-TMF2 was illuminated with the same number (720) of saturating flashes at varied intervals, and regeneration of $O_2$ evolution (Fig. 5A) and incorporated Mn (Fig. 5B) were plotted against the dark-time between flashes ($t_d$). The relative quantum efficiency for photoactivation of untreated $NH_2OH$-TMF2 was maximal at a $t_d$ of approx. 0.25 s. As mentioned earlier [11], the ascending and descending portions of the curve reflect the generation and decay, respectively, of the unstable intermediate in a photoactivation process requiring at least two quanta, and the half-times for the two processes in untreated $NH_2OH$-TMF2 were 125 ms and 1 s, respectively. These values are in good agreement with those previously reported [11,25,26]. When DEPC treatment was prolonged to 60 min, neither regeneration of $O_2$ evolution nor photoligation of $Mn^{2+}$ occurred, as shown in Fig. 1, under the steady-state conditions. Mild modification (10 min treatment) of the apo-complex modulated both half-times for ascending and descending portions mainly by enhancing the components with slower kinetics and the effect on the latter portion reflecting deactivation of the intermediate became pronounced. However, it remains unclear why the intermediate becomes more stable only in the mildly modified complex. Presumably, DEPC modification of histidine residues in PS II decreases the rate of generation of the intermediate and thereby affects the apparent stability of the intermediate to modulate the yield of $Mn^{2+}$ photoligation.

*Radiolabelling of PS II proteins by modification with [$^{14}$C]DEPC*

Fig. 6 shows the electrophoretograms and fluorograms of [$^{14}$C]DEPC-treated $NH_2OH$-TMF2 or TMF2. In $NH_2OH$-TMF2, radioactivity was found in CP47 apoprotein, CP43 apoprotein, D1, LHCP and a band at around 60 kDa, but a discrete fluorogram band of D2

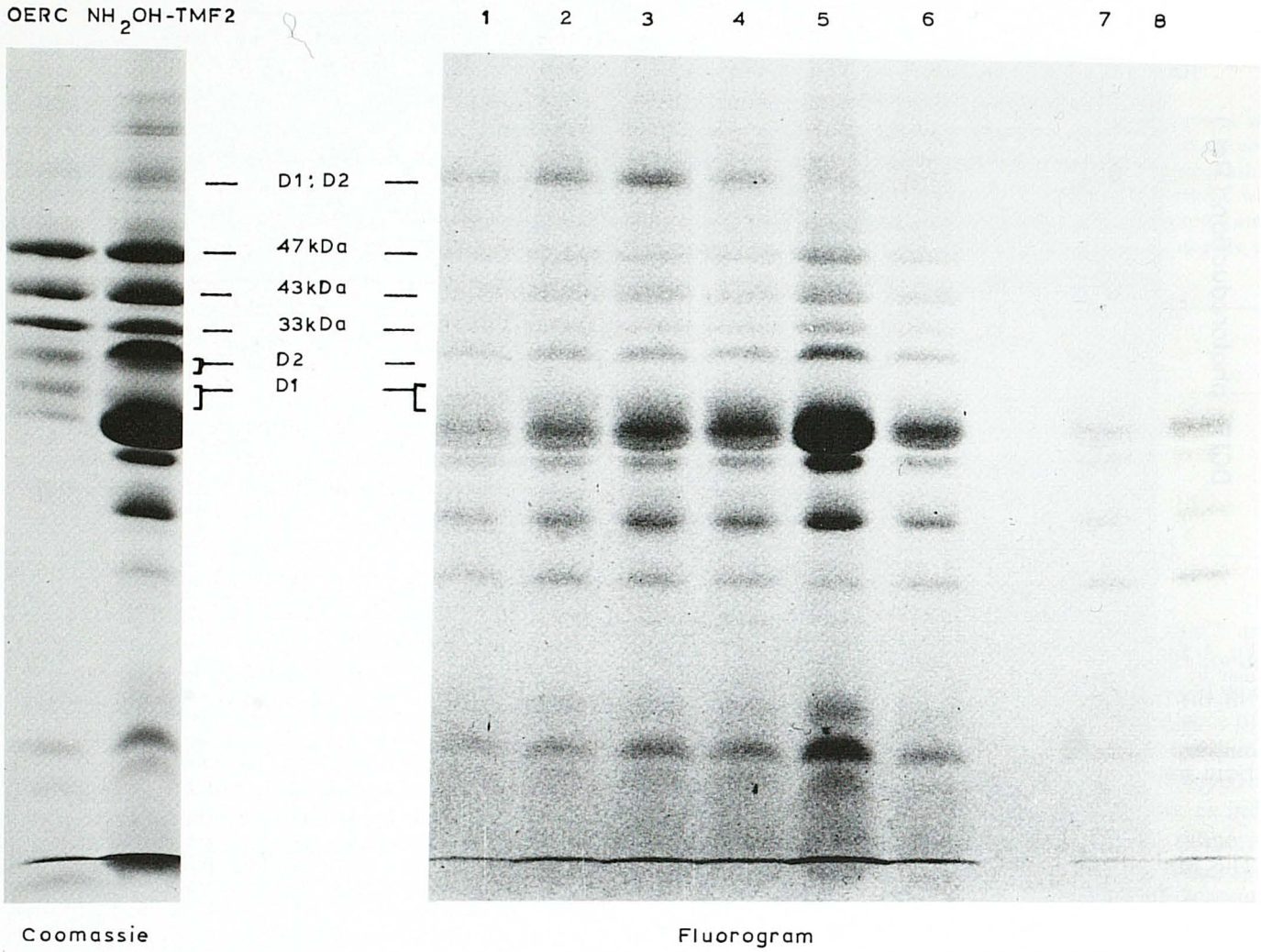


Fig. 6. Radiolabelling of $NH_2OH$-TMF2 and TMF2 with [$^{14}$C]DEPC. Left panel, Coomassie-stained $O_2$-evolving reaction center (OERC) and $NH_2OH$-TMF2. Right panel, fluorogram of radiolabelled $NH_2OH$-TMF2 and TMF2. Lanes 1–3, $NH_2OH$-TMF2 was treated with [$^{14}$C]DEPC in darkness for 15, 30 and 60 min, respectively; Lane 4, $NH_2OH$-TMF2 was treated with [$^{14}$C]DEPC in the presence of 1 mM $MnCl_2$ in darkness for 30 min; Lane 5, $NH_2OH$-TMF2 was treated with [$^{14}$C]DEPC during photoactivation under standard conditions (30 min); Lane 6, $NH_2OH$-TMF2 was photoactivated before [$^{14}$C]DEPC treatment in darkness for 30 min; Lanes 7 and 8, TMF2 was treated with [$^{14}$C]DEPC in darkness for 15 and 60 min, respectively.

was not obtained because of the presence of an unknown radiolabelled polypeptide at around 32 kDa (probably a part of LHCP (lanes 1–3)). In contrast, TMF2 was only weakly radiolabelled (lanes 7 and 8). This is in good agreement with the result that DEPC does not affect the $O_2$ evolution by TMF2 (Fig. 1). The addition of $Mn^{2+}$ during dark treatment with [$^{14}$C] DEPC did not much affect the radiolabelling pattern (lane 4), but its presence under weak illumination suppressed the incorporation of radioactivity into D1 and more clearly into the 60 kDa band (lane 5). This phenomenon is more clearly seen when $NH_2OH$-TMF2 was photoactivated: radiolabelling of both the 60 kDa band and D1 protein was remarkably blocked (lane 6). Based on the fact that the 60 kDa band is due to a heterodimer of D1 and D2 proteins [27], as evidence by immunoblotting with the antibodies raised against D1 and D2 proteins (Fig. 7), we conclude that the histidine residues specifically interacting with the functional Mn are located in D1 protein.

## Discussion

The imidazole ring of a histidine moiety functions as a ligand toward transition-metal ions in varieties of biologically important molecules. DEPC is known to react with protein amino-acid side-chains of histidine, lysine, tyrosine, cystein, serine and arginine as well as the $\alpha$-amino group of amino acids [12]. Although ethoxyformylation of tyrosine and lysine residues has been reported to proceed below pH 7, specific modification of histidine residues in PS II membranes is evidenced based on the following observations. (a) The DEPC-induced loss in photoactivation capability was

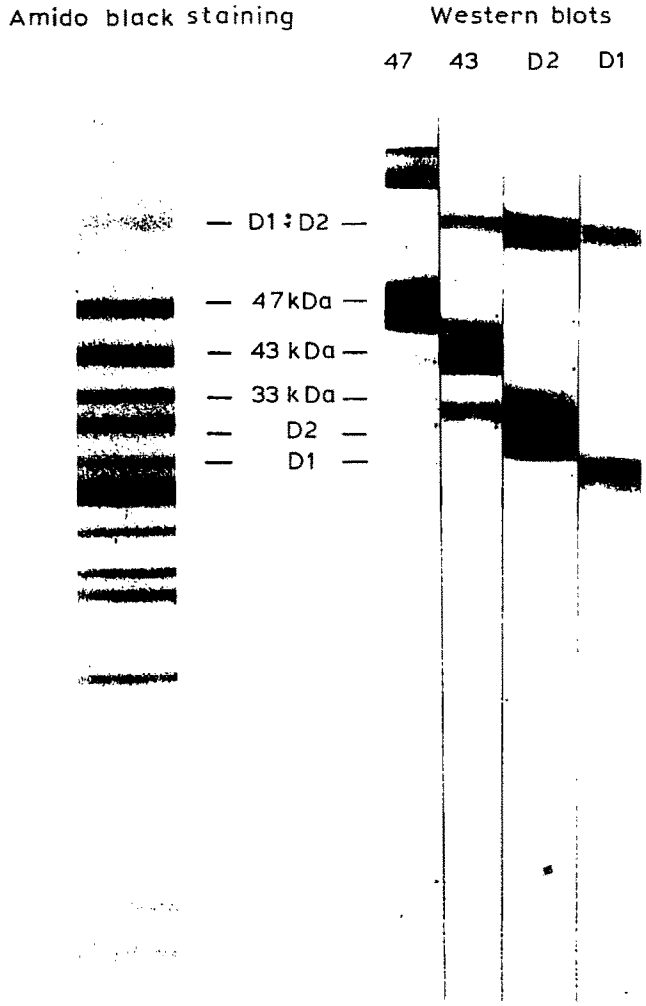


Fig. 7. Amido-black-stained $NH_2OH$-TMF2 (left panel) and immunodetection of CP47, CP43, D2 and D1 proteins in $NH_2OH$-TMF2 with Western blots (right panel).

almost completely reversed by $NH_2OH$ (Fig. 2). (b) The $pK_a$ value (= 6.9) estimated from the pH dependence of the rate constant of DEPC-induced inactivation (Fig. 3) coincided with the $pK_a$ values reported for modification of histidine residues in various enzymes [19,22]. (c) The second-order rate constant for inactivation (92.6 $M^{-1} \cdot min^{-1}$ at pH 6.5 at 20 °C; Fig. 3, inset) was relatively high as compared with those of other residues [20]. (d) There was little DEPC modification in the 33 kDa extrinsic protein which contains lysine, tyrosine, serine, cystein and arginine but no histidine [28], although the protein was weakly radiolabelled under weak light illumination (Fig. 6).

Chemical modification of histidine residues in $NH_2OH$-TMF2 caused a decrease in binding constant of exogenous $Mn^{2+}$ at its high-affinity binding sites (Fig. 4), accompanied with a decrease in capability of photoligation of $Mn^{2+}$ to the apo-complex (Fig. 1). On the other hand, the histidine modification did not affect photooxidation of $Mn^{2+}$ so much as photoactivation (Table I). It is thus inferred that DEPC modifies the histidine residues functional in $Mn^{2+}$ ligation, but the residue(s) is/are not involved in photooxidation of $Mn^{2+}$.

In a previous paper [11], we proposed a model for the two-quantum process to regenerate binuclear centers: (1) the first $Mn^{2+}$ is photooxidized by $Y^+$, the oxidized species of a proposed PS II electron donor, before its ligation with high affinity on the apo-complex; (2) ligation of the first $Mn^{3+}$ facilitates weak binding of the second $Mn^{2+}$ at a relatively slow rate (approx. 125 ms), probably involving some conformational changes in the Mn-binding sites; (3) photooxidation of the second $Mn^{2+}$ yields a binuclear center, which subsequently binds additional two $Mn^{2+}$ in the dark reactions to form a tetranuclear center with mixed Mn valency state. In line with this proposal, the results in Fig. 1 and Table I in this study are interpreted as indicating that histidine residues play a role in step (2). In fact, based on analysis of flash interval dependence of photoactivation (Fig. 5), the generation course of the unstable intermediate (the product of the step (2)) was retarded by histidine modification to apparently reduce the yield of the intermediate.

It remains to be solved which protein(s) or which amino-acid residues are ligated with Mn in the water-oxidizing complex. However, we can at least restrict the sites for $Mn^{2+}$-binding/ligation on PS II intrinsic proteins based on the following results: (1) the water-oxidizing complex retains most of the manganese after removal of all the extrinsic proteins by $CaCl_2$ [29] or NaCl/urea [30]; and (2) full photoligation of $Mn^{2+}$ can be attained in the absence of all the extrinsic proteins [10,11]. Recently, an $^{125}I$-labelling study [31] revealed that D1 protein is iodinated by $I^-$, which functions as an electron donor for the incompetent PS II membranes, and the iodination is competitively inhibited by $Cl^-$. Based on these and other data, they suggested that the active center of the Mn complex or PS II secondary electron donor, Z, is located on/near D1 protein.

Klein and colleagues [4] have gained an insight into the ligands of Mn at the active center. Based on analysis of EXAFS spectra, they claimed that a Mn atom at a cluster consisting of two binuclear centers in close proximity (approx. 2.7 Å) contains two atoms such as C, N or O at 3.0 at 3.3 Å, possibly originating from an imidazole ring of histidine residues. In this study, we showed an almost complete loss of photoactivation capability by DEPC modification (Fig. 1) and its protection afforded by the presence of exogenous $Mn^{2+}$ under weak light illumination (Table II). These imply the existence of a histidine residue essential for $Mn^{2+}$ ligation in the intact water-oxidizing complex.

The radiolabelling experiments with [$^{14}C$]DEPC (Fig. 6) gave us a more concrete view about the protein that carries the histidine residue responsible for $Mn^{2+}$ li-

gation. Among the [$^{14}$C]DEPC-labelled proteins, the radiolabelling of D1 protein was almost completely blocked by the presence of $Mn^{2+}$ under weak light illumination. This is completely parallel with the substrate protection found for DEPC-induced loss of photoactivation capability (Table II), and suggests that the histidine residues responsible for Mn ligation are located on D1 protein. In view of the symmetric relationships between D1 and D2, labelling of D2 is anticipated. However, radiolabelling of D2 protein could not be detected clearly, because of overlapping of nonspecifically labelled proteins in the 25–33 kDa region. Further investigation with a more purified reaction-center complex depleted of LHCP is needed for this purpose.

The results in this study provide no information as to the location on D1 (and possibly D2) protein of the histidine residue(s) responsible for Mn ligation. The possible sites may, however, be speculated in the following way. D1 and D2 proteins of spinach contain ten and eight histidine residues, respectively. Among these, three of the six symmetrical residues (His-198, His-215 and His-272) on helices IV and V in both D1 and D2 subunits are synergetically involved in binding P-680, $Q_A$ or $Q_B$, and Fe, respectively. In this analogy, other symmetrical histidine residues located in the loops between the membrane-spanning helices contribute to the structural integrity of the water-oxidizing complex by providing ligands to Mn. This assumption is invoked by recent findings as follows. (1) The PS II donor activity photoinhibited by weak illumination was recovered in parallel with synthesis of not only D1 but also D2 proteins [32–34], suggesting that both proteins function cooperatively in photooxidation of $Mn^{2+}$ and/or ligation of $Mn^{\geq 3+}$. (2) The reaction-center complex consisting of only three different proteins, D1, D2 and cytochrome *b*-559 (Ref. 35), is capable of photooxidizing $Mn^{2+}$ (unpublished data) or DPC [27], and possesses the high-affinity $Mn^{2+}$-binding sites (Seibert, M., Tamura, N. and Inoue, Y., unpublished data), indicative of existence in this complex of the site for photooxidation/photoligation of $Mn^{2+}$. (3) $^{125}$I-Labelling experiments [15,36] revealed that D1 and D2 proteins contain the vicinity of Z and D domains on the donor side of PS II, respectively. Furthermore, recently, Debus et al. [37] concluded that D is Tyr-160 of D2 by employing both site-directed mutagenesis and ESR signal detection, and suggested by analogy with this that Z corresponds to Tyr-161 of D1. As for the spatial relationship between the Mn ligation sites and D or Z, Itoh et al. [38] have suggested, from the effects of spin-relaxing reagent on power saturation profiles of Sig $II_s$ spectra, that the Mn-binding site is located on the inner surface close to D or Z (13–14 Å distant). According to the model of folding of D1 and D2 predicted by Trebst [39], two histidines, His-190 and His-337, are located inside thylakoids, while His-118 is situated on helix II. Therefore, we speculate that the site(s) of DEPC modification of histidine residues to cause a loss in photoactivation capability is His-190 or/and His-337 of at least D1 protein. However, there is no evidence at present that D1 and D2 cooperatively possess the Mn ligation sites or that only the symmetrical histidines on D1 (and D2) functions in ligation of Mn. Thus, we cannot exclude the possibility of involvement of asymmetrical histidines (His-92/His-332) on D1 protein as a Mn ligand.

Several amino-acid residues must be assumed for full ligation of four Mn. Obviously, the number of histidine residues in D1 (or D2) protein is not enough to provide sufficient numbers of ligands for all of these Mn atoms. In preliminary experiments, we have observed a $Mn^{2+}$-specific substrate protection in modification with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, a water-soluble carboxyl modifier. This suggests that carboxyl groups on PS II intrinsic proteins are also involved in Mn ligation.

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