Chemical Modification of Amine Groups on PS II Protein(s) Retards Photoassembly of the Photosynthetic Water-Oxidizing Complex†

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ABSTRACT: Four Mn atoms function as catalysts in the water-oxidizing complex located on the oxidizing side of PS II. We have studied the involvement of amine groups of the PS II proteins in photoligation of Mn^{2+} to the apo water-oxidizing complex, using the combined techniques of photoactivation and chemical modification with the modifiers methyl acetimidate (MAI), acetic acid *N*-hydroxysuccinimide ester (NHS), and 2,4,6-trinitrobenzenesulfonic acid (TNBS). Chemical modification of hydroxylamine-treated PS II core complexes decreased their capacity for restoration of oxygen evolution and photoligation of Mn^{2+} to the apo water-oxidizing complex (WOC), but did not affect their electron transfer activity in the vicinity of PS II. The number of functional high-affinity Mn-binding sites, but not of low-affinity sites, was significantly modulated by chemical modification. Kinetic analysis of photoactivation with the repetitive flashes revealed that the intermediate generated during a photoactivation process was destabilized by the chemical modification. To identify which proteins possess the amine groups involved in ligation of functional Mn, we examined the difference in NHS biotinylation between PS II core complexes with and without the Mn cluster. NHS biotinylation resulting in altered ligation of functional Mn apparently occurred on three proteins: an antenna chlorophyll binding protein (CP47), a light-harvesting chlorophyll protein (CP29), and another chlorophyll binding protein (PS II-S). Of these proteins, only the Mn-dependent biotinylation of CP47 was found to occur independently of the application of an NHS-masking concentration before removal of the functional Mn. These results suggest that lysyl residues of CP47, and perhaps also CP29 and PS II-S, function in direct photoligation of Mn^{2+} to the apo WOC.

In cyanobacteria and higher plants, the primary process of conversion of light to chemical energy takes place in two photosystems of the thylakoid membranes, PS I and PS II.[1] In PS II, a reaction center chlorophyll (P680) absorbs photons and oxidizes the PS II secondary donor (Z), resulting in the accumulation of strong oxidizing equivalents and the subsequent water oxidation (*1*−*3*). The water-oxidizing reaction is catalyzed by manganese atoms, which are clustered and harbored within the PS II multifunctional complex consisting of the two reaction center proteins, D1 and D2, the chlorophyll-binding proteins, CP47 and CP43, Cyt b_{559}, three PS II extrinsic proteins, and a number of low-molecular mass intrinsic proteins. Spectroscopic studies with EXAFS, ESR, and ESSEM have revealed that the Mn cluster consists of two di-μ_2-oxo dimers which are bridged by one mono-μ_2-oxo and two carboxylate bridges (*1*, *4*).

The assembly of the Mn cluster requires light in a process termed photoactivation of the water-oxidizing complex (WOC). In this process, two Mn^{2+} ions are oxidized with the absorption of two photons, and are bound and ligated to the PS II apo complex as follows (*5*−*9*). The photoactivation process proceeds via two successive photoreactions, with an unstable intermediate that may involve Mn^{3+} being formed by the first photoreaction. Another Mn^{2+} is photooxidized to Mn^{3+} by a second photoreaction, resulting in the generation of a stable Mn dimer with higher oxidation states. Subsequent ligation of two more Mn^{2+} ions probably takes place in darkness. Cofactors such as Ca^{2+} and Cl^- are required for photoactivation as well as for S-state transition (*5*, *6*, *9*, *10*). Although the Mn cluster has a complex structure, the experimental procedure for photoactivation is quite simple. Chloroplasts and PS II membranes that are depleted of only functional Mn can recover water-oxidizing capability if they are incubated in the presence of Mn^{2+} and Ca^{2+} under weak illumination at room temperature and at the appropriate pH (*5*, *11*).

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[1] Abbreviations: CAB, chlorophyll *a*/*b* binding protein; CBB, Coomassie Blue R-250; CP47 and CP43, antenna chlorophyll−protein complexes of PS II; DCIP, 2,6-dichlorophenolindophenol; DEPC, diethyl pyrocarbonate; DPC, 1,5-diphenylcarbazide; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; LHC, light-harvesting chlorophyll proteins; MAI, methyl acetimide; NHS, *N*-hydroxysuccinimide ester; PS II, photosystem II; TNBS, 2,4,6-trinitrobenzenesulfonic acid; WOC, water-oxidizing complex; Yz, redox-active tyrosine 161 of the D1 protein.

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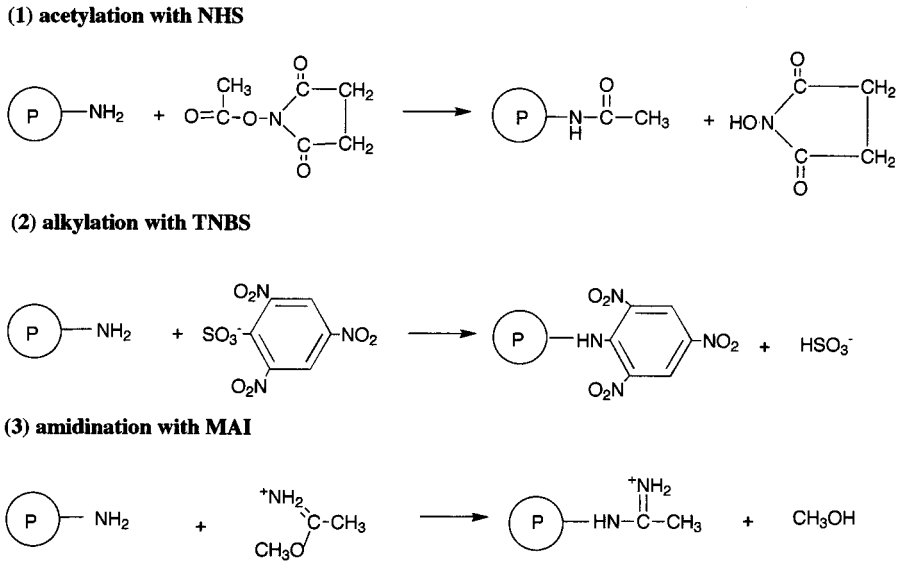
Chart 1



Precise identification of the proteins ligating functional Mn still remains to be achieved. On the basis of Mn coordination chemistry, possible candidate ligands to the Mn complex have been proposed to be carboxyl, alkoxo, phenoxo, and imidazole groups in amino acids (*12*). The cluster may be ligated with approximately six ligands in addition to water molecules, Cl^-, and Ca^{2+} (*13*). Spectroscopic and site-directed mutagenesis studies (*1*, *14*−*17*) inferred that carboxyl groups and histidine residues on the lumenal side of the D1 and D2 proteins are ligands to the Mn complex, including seven acidic amino acid residues and two histidyl residues on the D1 protein and one acidic amino acid residue on the D2 protein. Histidyl and acidic amino acids residues were also implicated in (photo) ligation of manganese to the apo WOC by use of combined techniques, including photoactivation and chemical modification (*18*−*20*). Treatment of PS II membranes depleted of functional Mn with diethyl pyrocarbonate (DEPC), a modifier of histidine residues, specifically modified histidine residues on the D1 protein, and resulted in a loss of photoactivation capability of PS II membranes. It was also found that chemical modification of carboxyl residues of the D1 protein with 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) abolished photoactivation capability. Seibert and co-workers (*21*−*24*) and Magnusson and Andreasson (*25*) also reported that chemical modification of the apo Mn complex with EDC and DEPC caused partial loss of the high-affinity Mn-binding sites and concluded that specific histidyl and carboxyl residues of the D1 protein function in binding and/or ligation of Mn.

According to a model of the Mn cluster presented by Cinco et al. (*13*), the Mn cluster may require several additional ligands beyond carboxyl and imidazole groups, i.e., oxygen atoms bridged by Ca^{2+} and Cl^-. Chlorophyll binding proteins CP47 and CP43 have also been reported to be involved in ligation of the Mn cluster (*26*, *27*). In this study, we reexamined the involvement of amine groups in ligation of manganese to the complex. Amine groups are known to be potential ligands for metal ions (*12*), although this function is relatively poor at physiological pH. Chemical modification of the Mn-depleted PS II core complexes with different types of amine groups modifiers was found to significantly impair photoligation of Mn^{2+}. We further identified proteins which are specifically modified by NHS-biotin in a manner consistent with a role in photoactivation.

MATERIALS AND METHODS

Isolation of PS II Preparations. Oxygen-evolving PS II membranes were obtained from spinach leaves as described in ref *5*. Oxygen-evolving PS II core complexes were isolated by treating the PS II membranes with 50 mM *n*-octyl *β*-D-glucopyranoside according to the method of Ghanotakis et al. (*28*). Treatment of the membranes with 0.8 M Tris (pH 8.0) or 2 mM NH_2OH (pH 6.5) was performed basically as described by Tamura and Cheniae (*5*). These preparations were used either immediately or after storage at −80 °C.

Chemical Modification. Unless otherwise noted, chemical modifications of PS II membranes depleted of functional Mn (250 *μ*g of Chl/mL) by methyl acetimidate (MAI, 500 mM), 2,4,6-trinitrobenzenesulfonic acid (TNBS, 1 mM), and *N*-hydroxysuccinimide acetate (NHS, 20 mM) were carried out at the specified temperature (4 or 20 °C) and pH (7.5, 9.0, or 9.5) for 40−80 min. The membranes were then washed three times in buffer A [50 mM Mes-NaOH (pH 6.5), 20 mM NaCl, and 0.4 M sucrose] and resuspended in the same buffer. The chemical modification reactions involving α- and *ϵ*-amino groups of the proteins with NHS, MAI, and TNBS are shown in Chart 1: (1) acetylation with NHS, (2) alkylation with TNBS, and (3) amidination with MAI. As indicated in Chart 1, chemical modification with MAI results in a positively charged product at physiological pH, while the products formed with TNBS and NHS are neutral. TNBS and NHS can react with cysteine thiols and with tyrosine, respectively. In the former case, the resultant thiol derivatives are unstable at the alkaline pH, and in the latter, the acetyl group attached to the tyrosine residues can be removed by the subsequent treatment with NH_2OH at high concentrations (>0.2 M).

NHS-Biotin Treatment. The treatment of the PS II preparations with NHS-biotin was performed basically as described by Frankel and Bricker (*29*). Membranes equivalent to 100 *μ*g of Chl/mL were incubated with 0.3 mg/mL NHS-biotin

at 20 °C for 40 min in darkness, and were then washed three times with a large amount of buffer A. In case of the masking treatment with NHS before NHS biotinylation, membrane preparations equivalent to 50 μg of Chl/mL were pretreated with NHS at 10−100 mM for 10 min on ice. For detection of NHS-biotinylated proteins, the NHS-biotin-treated membranes were analyzed by SDS−PAGE, electroblotted onto PVDF membranes (Immobilon-P, Millipore Co.), and then developed with an avidin-conjugated HRP and either an HRP color kit (Bio-Rad) or enhanced chemiluminescence reagents (ECL; Amersham).

Photoactivation Procedure. Chemically modified membranes were subjected to photoactivation as described by Tamura and Cheniae (*5*). Unless noted otherwise, NH_2OH-treated PS II membranes (200 μg of Chl/mL) or PS II core complexes (50 μg of Chl/mL) were incubated with buffer A containing 1 mM $MnCl_2$, 50 mM $CaCl_2$, and 100 μM DCIP, at 22 °C for 30 min under weak light (at a PPFD of 30 μE m^{-2} s^{-1}).

DCIP Photoreduction. DCIP photoreduction was assayed by monitoring the changes in absorbance at 590 nm using an extinction coefficient of 15 mM^{-1} cm^{-1}, with a double-beam, dual-wavelength spectrophotometer (Shimadzu model UV-300). The standard medium for measurement consisted of buffer A that contained 50 μM DCIP and either 500 μM DPC or 2 mM $MnCl_2$. To evaluate the high-affinity Mn-binding sites in PS II preparations, we employed the hydrogen peroxide-supported DCIP photoreduction assay as described by Inoue and Wada (*30*); the reaction mixture consisted of buffer A containing 3 mM hydrogen peroxide, 50 μM DCIP, PS II preparations equivalent to 5 μg of Chl/mL, and a given concentration of $MnCl_2$.

Other Procedures. Oxygen-evolving activity was measured polarographically with a Clark-type electrode (Rank Brothers) at 22 °C in buffer A containing 10 mM $CaCl_2$, 1 mM ferricyanide, and 250 μM phenyl-*p*-benzoquinone. Ferricyanide alone was used as an electron acceptor for the assay of oxygen-evolving activity with PS II core complexes.

Mn determinations were made in an inductively coupled plasma mass spectrometer (Yokogawa model PMS-2000), following complete digestion of samples with a mixture of concentrated HNO_3 and 70% $HClO_4$ (9:1, v/v). The digestion procedure was carried out according to the method described in ref *31*, except that 10 mM HNO_3 without $HClO_4$ was used for rinsing the ashed samples. SDS−PAGE analyses were carried out using 5% stacking and 12.5% polyacrylamide running slab gels.

RESULTS

Inactivation of the Photoactivation Capacity of the Apo Water-Oxidizing Complex by Chemical Modification of Amine Groups. The level of DCIP photoreduction supported by DPC in NH_2OH-treated PS II core complexes (NH_2OH−PS II core), which were depleted of functional Mn, decreased only slightly to 70−90% of the control by chemical modification with NHS (20 mM, pH 7.5), TNBS (1 mM, pH 9.5), or MAI (500 mM, pH 9.0) within the time that was examined (Figure 1). The Mn^{2+}-supported DCIP photoreduction was also not inhibited; in fact, it was even slightly enhanced. On the other hand, the capacity for photoactivation was drastically decreased to 10−20% of the control after

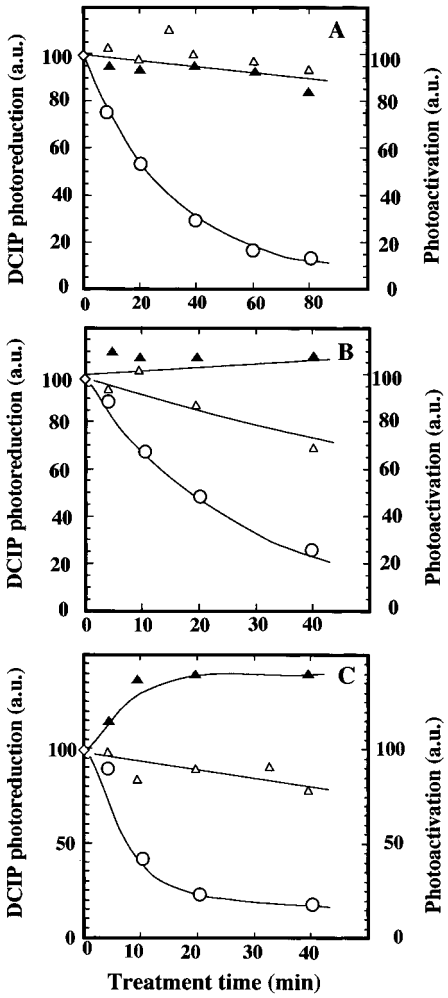


FIGURE 1: Effects of chemical modification of amine groups on DCIP photoreduction supported by Mn^{2+} or DPC, and on photoactivation capability in NH_2OH-treated PS II core complexes (NH_2OH−PS II core). Results obtained with NHS-, TNBS-, and MAI-treated preparations are shown in panels A−C, respectively. Empty and filled triangles show DCIP photoreduction donated by DPC and Mn^{2+}, respectively, whereas empty circles show photoactivation. Chemical modification was performed for the times indicated under the conditions described in Materials and Methods.

treatment for 60 min. Similar results were obtained with NH_2OH-treated PS II membranes (data not shown). These results suggest that treatment with the chemical modifiers specifically modified a light-required assembly process of the WOC with little decrease in PS II electron donor activity.

The three chemical modifiers used for Figure 1 are known to react with both α- and ϵ-amino groups. Thus, chemical modification of the N-terminal amino groups could be responsible for their effects on photoligation of Mn^{2+} to the apo WOC. However, this possibility appears to be unlikely on the basis of the following reasons. (1) Chemical modification of the PS II membranes with functional Mn had little effect on O_2 evolution activity (data not shown), and (2) N-terminal amino groups on the major proteins of PS II core are located in the stromal side. Of the chemical modifiers we employed, NHS exhibits the least specificity for amino groups; it can also react with tyrosine, serine, and threonine (*32*). We thus asked whether NH_2OH would abolish NHS-induced inactivation of photoactivation, since this reagent is known to remove the *O*-acetylated groups from the chemically modified proteins. Incubation of modified PS II

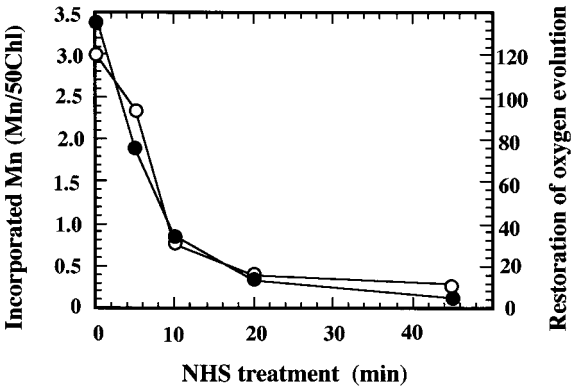


FIGURE 2: Effects of NHS treatment on photoligation of Mn^{2+} to the WOC. Empty and filled circles indicate Mn abundances (Mn/50Chl) and rates of DCIP photoreduction (micromoles of DCIP per milligram of Chl per hour), respectively, obtained with NH_2OH−PS II core samples that were treated with NHS for the indicated time and subsequently photoactivated.

samples with 0.3 M NH_2OH at 4 °C for 3 h did not induce deacetylation (data not shown), indicating that NHS specifically modified amine groups under our experimental conditions.

On the basis of the previous findings with the chemical modifiers for histidyl (*18*) and carboxyl groups (*20*), we expected that the loss of capacity for photoligation of Mn^{2+} seen in Figure 1 resulted from alteration of the high-affinity Mn-binding sites. However, the results could also be explained if the chemical modification caused a cessation of the transitions through the S-state. To examine this possibility, we examined Mn abundance after photoactivation of the NHS-treated NH_2OH−PS II core. We found a decrease in Mn abundance that paralleled that of oxygen-evolving activity as the extent of NHS modification increased (Figure 2). These results suggest that the observed inhibition of the restoration of oxygen-evolving activity by chemical modification is likely due to a loss of the ability of the apo WOC to photoligate Mn.

Effects of the chemical modification on the intactness of the high-affinity Mn-binding sites are shown in Figure 3. Previous studies using chemical modifiers (*18*−*20*) indicated that chemical modification of histidyl and carboxyl residues on a PS II protein(s) modulates the binding of exogenous Mn^{2+} to the high-affinity Mn-binding sites, which is accompanied by a loss of capacity for photoactivation. In this study, we employed DCIP photoreduction supported by Mn^{2+} at sub-micromolar concentrations coupled with hydrogen peroxide as an indicator of the intactness of the high-affinity Mn-binding sites. In this assay, Mn^{2+} can donate an electron to the secondary donor of PS II, Yz, but only with relatively low efficiency (the half-maximal concentration for Mn^{2+} donor activity is a sub-millimolar concentration). In the presence of hydrogen peroxide, the ability of Mn^{2+} to donate electrons to PS II improves dramatically, inferring that reduction of photooxidized Mn ions by hydrogen peroxide stimulates the turnover of Mn^{3+} to Mn^{2+} at the Mn-binding sites (*30*, *33*). Figure 3 shows the biphasic dependence of Mn^{2+}-supported DCIP photoreduction on exogenous Mn^{2+} concentration, as reported by Inoue and Wada (*30*). This was quantitated by determining the Michaelis−Menten constants from the double-reciprocal plots in the inset of Figure 3. These parameters (K_m) reflect apparent dissociation constants

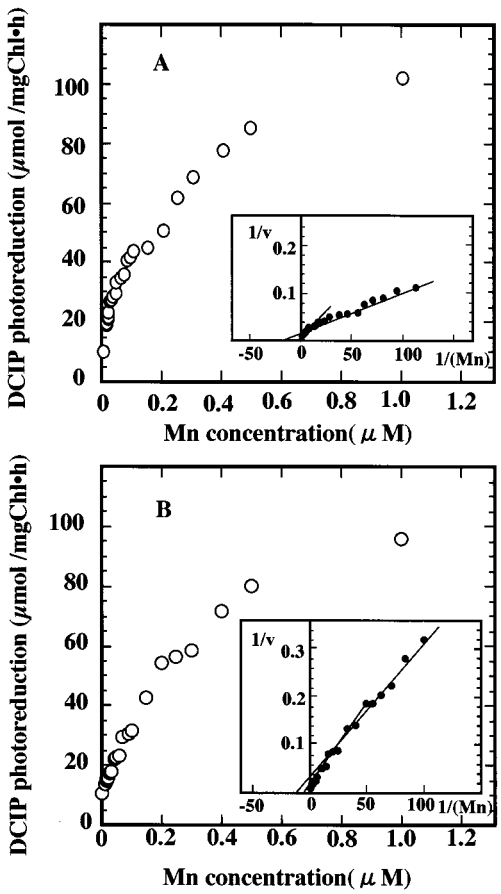


FIGURE 3: Dependence of DCIP photoreduction on Mn^{2+} concentration in the presence of hydrogen peroxide in untreated and NHS-treated NH_2OH−PS II core samples: (A) NH_2OH−PS II core samplesand (B) NH_2OH−PS II core samples which were chemically modified with NHS. The insets show the double-reciprocal plots of the rates of DCIP photoreduction as a function of Mn concentration.

for dissociation of exogenous Mn^{2+} from the Mn-binding sites; K_{m1} and K_{m2} are likely to reflect the intactness of the high- and low-affinity Mn-binding sites, respectively. We obtained values of 0.03 and 0.25 μM for K_{m1} and K_{m2}, respectively, in unmodified preparations, which are similar to those reported with Mn-deficient PS II membranes (*30*). In NHS-treated preparations, increasing the extent of chemical modification led to an increase in the value of K_{m1} from 0.03 to 0.25 μM, while the values of K_{m2} remained constant (Figure 4A). Interestingly, following treatment for 60 min, the value of K_{m1} was almost identical to that of K_{m2}. Similar results were obtained in preparations treated with TNBS and MAI (Figure 4B,C). These results indicate that chemical modification of amine groups specifically alters the affinity of Mn^{2+} for the high-affinity Mn-binding sites.

Dependence of the Yield of Photoactivation on the Dark Time between Flashes. As proposed previously (*5*, *7*−*9*), photoactivation of the apo WOC may proceed as shown in Scheme 1. In this model, an inactive form, D, of the WOC depleted of functional Mn is converted to an intermediate, L_1, by the first photoreaction, wherein Mn^{2+} is photooxidized to Mn^{3+} and then bound to the high-affinity Mn-binding sites. L_1 is subsequently transformed to L_2 with the rate constant k_A in darkness. Following the second photoreaction, L_2 is converted to L_3, from which an active center equipped with functional Mn is formed in the dark process. From here,

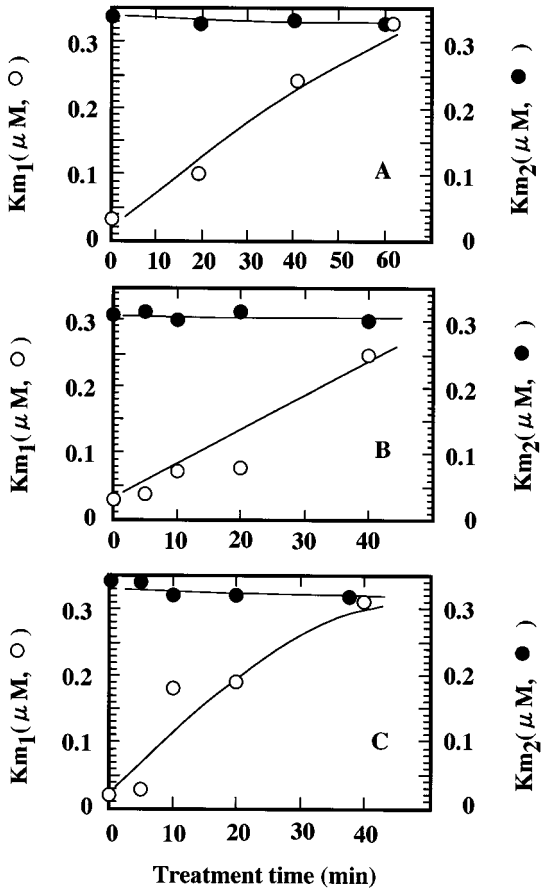
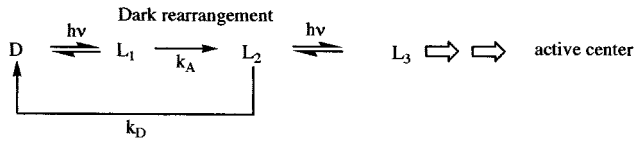


FIGURE 4: Effects of chemical modification on the Michaelis−Menten constants (K_{m1} and K_{m2}) for Mn^{2+}-supported DCIP photoreduction in the presence of hydrogen peroxide in NHS-treated (A), TNBS-treated (B), and MAI-treated (C) NH_2OH−PS II core samples.

Scheme 1



unless the second photoreaction takes place in an appropriate time frame, L_2 will decay to D with a rate constant k_D. Therefore, as previously reported in ref *5*, the population of L_2 after the *n*th flash is calculated with the following equation:

$$[L_2]_n = \phi[k_A/(k_D - k_A)][D]_{n-1}(e^{-k_A t_d} - e^{-k_D t_d}) \quad (1)$$

where $[D]_{n-1}$ is the population of D after the $(n-1)$th flash, ϕ is the quantum efficiency of the conversion of D to L_2, and t_d is the dark time between flashes. The population of the active Mn clusters generated by the *n*th flash should be proportional to that of $[L_2]_n$ determined by the above equation. Since ϕ is very low (<0.01) and $[D]_n$ after 750 flashes is estimated to be 80−90% of that before the first flash, we can approximate eq 1 as follows:

$$[L_2]_n \approx [L_2]_1 = \phi[k_A/(k_D - k_A)][D]_0(e^{-k_A t_d} - e^{-k_D t_d}) \quad (2)$$

where $[D]_0$ and $[L_2]_1$ are the population of D and L_2 before and after the first flash, respectively. Assuming the number of the Mn clusters generated by *n* flashes is proportional to $[L_2]_1$, we will estimate the rate constants for the transitions from L_1 to L_2 (k_A) and from L_2 to D (k_D) from eq 2. Miller and Brudvig (*7*) also deduced a similar equation from the above scheme, except that they further introduced rate constants for the transition from D to L_1 and its back reaction, from L_1 to D.

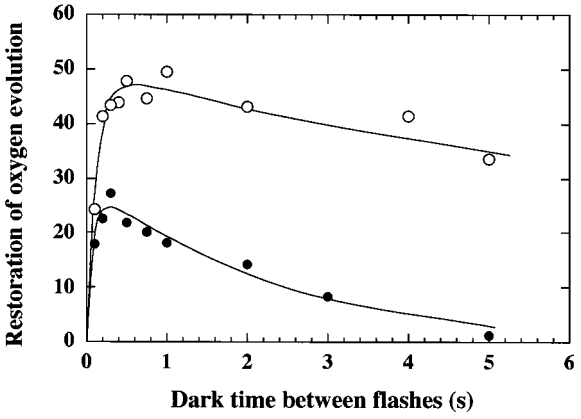


FIGURE 5: Dependence of photoactivation on dark intervals between flashes in untreated and 10 mM NHS-treated NH_2OH−PS II core samples (○ and ●, respectively). The core complexes were photoactivated by 750 flashes with the indicated intervals. The curves were obtained as fits to the data using eq 2.

To examine the stability of L_2 in the NHS-modified preparations, we studied the dependence of photoactivation yield on the dark time between flashes, which was varied from 100 ms to 5 s (Figure 5). Since a pH of 5.5 is optimum for photoactivation of PS II core complexes depleted of the functional Mn by steady-state illumination or flashes, we opted for this pH for the experiment. In untreated preparations, k_A and k_D were 7.6 and 0.06 s^{-1}, respectively, whereas they were 11 and 0.43 s^{-1}, respectively, in core complexes treated with 10 mM NHS. Chemical modification by NHS caused a dramatic increase in k_D, but not in k_A, suggesting that the modification makes L_2 more unstable and stimulates the inactivation of L_2 to D. This result is quite different from that previously reported with DEPC- or EDC-treated PS II membranes (*18*, *20*), in which L_2 became stable after the chemical modification.

Effects of NHS Modification on Rebinding of the 33 kDa Protein to the Membranes. Frankel and Bricker (*29*) reported that rebinding of the 33 kDa extrinsic protein to the $CaCl_2$-washed PS II membranes was inhibited by the NHS modification. They concluded, drawing also on results from site-directed mutagenesis (*34*, *35*), that the E-loop containing lysyl residues on CP47 is specifically modified by NHS and is involved in association of the 33 kDa extrinsic protein to the membranes. Thus, we examined whether NHS modification caused a decrease in the binding capacity of the 33 kDa protein under our experimental conditions. In a previous study (*36*), the 33 kDa protein was observed to rebind to PS II membranes at a stoichiometry of 2 mol of protein per mole of PS II, and this number was independent of the presence of functional Mn. To saturate the binding sites, we incubated the 33 kDa protein in a 10:1 molar ratio with PS II core complexes depleted of functional Mn.

We employed two types of PS II samples in the experiment described in the legend of Figure 6: Tris-treated PS II core complexes (Tris−PS II core) that have neither functional Mn

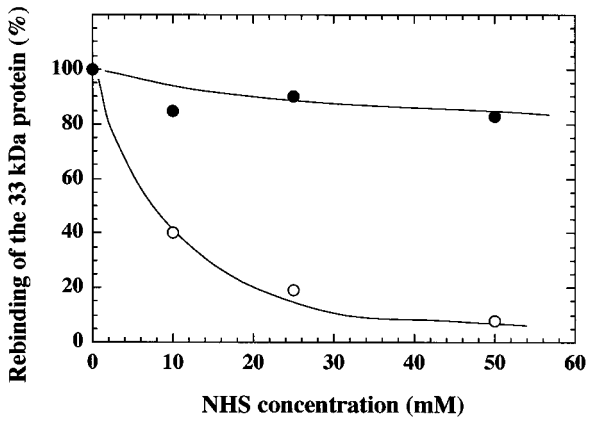


FIGURE 6: Rebinding of the 33 kDa extrinsic protein to NHS-treated PS II core complexes. Empty and filled circles are results obtained with NHS-treated Tris−PS II core and NHS-treated NH_2OH−PS II core samples that were subsequently treated with 1.2 M $CaCl_2$. PS II core complexes were incubated with the 33 kDa protein, washed, and then analyzed via SDS−PAGE. Gels were stained with CBB, and band intensities were measured densitometrically.

nor the 33 kDa protein and NH_2OH-treated PS II core complexes (NH_2OH−PS II core) that have no functional Mn but still retain >80% of the 33 kDa protein. These core complexes contained neither the 23 nor 17 kDa extrinsic protein. We found a large difference in the rebinding capability of the 33 kDa protein between the two NHS-modified preparations. As previously reported (*29*), NHS modification of Tris-treated PS II core complexes caused a significant decrease in the level of the subsequent rebinding of the 33 kDa protein to the membranes [Figure 6 (○)]. In a similar experiment performed with NH_2OH-treated core complexes, the samples were treated first with NHS and subsequently with 1.2 M $CaCl_2$ to release the 33 kDa protein [Figure 6 (●)]. The capacity of these samples for rebinding the 33 kDa protein was affected only slightly by the NHS treatment. These results indicate that binding sites for the 33 kDa protein remained intact even after NHS treatment with NH_2OH-treated PS II core samples. Thus, we may exclude the possibility that loss of the 33 kDa binding sites by chemical modification is responsible for the loss of Mn^{2+} photoligation to the apo WOC under our experimental conditions.

Identification of the Protein(s) Specifically Modified with NHS. The sites modified by NHS in PS II core complexes were analyzed with NHS-biotin (Figure 7). The pattern of proteins labeled with NHS-biotin (lanes 1 and 2 in Figure 7B) was similar to that observed in the gel stained with CBB (lanes 2 and 3 in Figure 7A). NHS biotinylation caused some bands to appear diffuse and shifted proteins to higher-molecular mass regions (lanes 5 and 6 in Figure 7A). Several bands disappeared with NHS chemical modification, probably due to a significant loss of positively charged groups that could interact with the negatively charged CBB molecules (*37*).

To identify the specific NHS modification site(s) in PS II proteins, $CaCl_2$−PS II core complexes ($CaCl_2$−PS II core, which are samples depleted of PS II extrinsic proteins but retaining functional Mn) were masked with NHS at a given concentration and then labeled with NHS-biotin before and after removal of functional Mn. Two major bands, designated bands II and III in Figure 7, were biotinylated in the presence

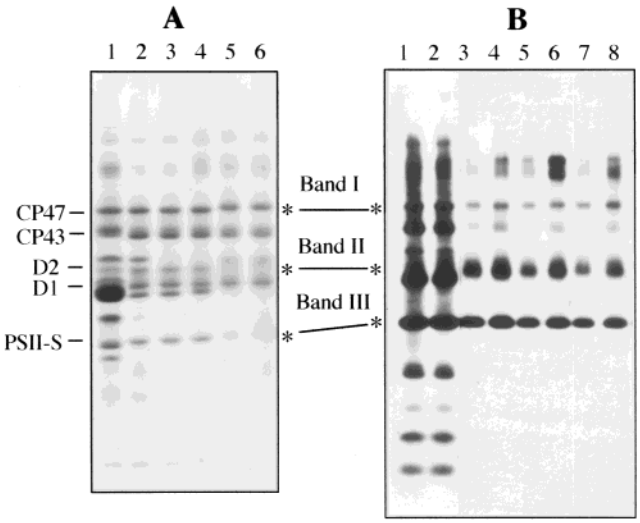


FIGURE 7: SDS−PAGE with untreated and NHS-biotinylated PS II core complexes. Panels A and B show CBB staining and NHS-biotinylated proteins detected by avidin, respectively. Panel A: lane 1, PS II membranes; lane 2, PS II core complexes; lane 3, $CaCl_2$−PS II core complexes; lane 4, NHS-biotinylated $CaCl_2$−PS II core; lane 5, 10 mM NHS-treated $CaCl_2$−PS II core complexes that were labeled with NHS-biotin; and lane 6, 10 mM NHS-treated $CaCl_2$−PS II core complexes that were treated with NH_2OH and then labeled with NHS-biotin. (B) NHS biotinylation was performed with the following preparations: PS II core complexes (lane 1), $CaCl_2$−PS II core complexes (lane 2), $CaCl_2$−PS II core complexes that were treated with NHS at 10 (lane 3), 50 (lane 5), and 100 mM (lane 7), and $CaCl_2$−PS II core complexes that were treated with 2 mM NH_2OH to delete functional Mn, following treatment with NHS at 10 (lane 4), 50 (lane 6), and 100 mM (lane 8).

of functional Mn in NHS-masked $CaCl_2$−PS II core samples (lanes 3, 5, and 7). Most other PS II proteins were not heavily biotinylated, although some minor biotinylation was observed for proteins larger than 60 kDa. NHS biotinylation of $CaCl_2$-treated PS II cores that had been further treated with NH_2OH to remove functional Mn displayed an additional band, designated as Band I, the level of biotinylation of which appeared to increase upon Mn removal (compare lanes 4, 6, and 8 with lanes 3, 5, and 7). From these results, it is postulated that Bands I, II, and/or III may be involved in ligation of Mn^{2+} by the WOC.

On the basis of its position in the gel, Band III was identified as PS II-S, a protein with an apparent molecular mass of 22 kDa (*38*, *39*). To identify Bands I and II, we probed Western blots with antibodies raised to CP47, CP43, D2, D1, and LHC (Figure 8). Band I was clearly shown to be CP47. The bands in the higher-molecular mass regions of the blot cross-reacted with anti-CP47, suggesting that they are cross-linked products of CP47. Band II, which was diffuse in the molecular mass region around 32 kDa, was located between the D1 and D2 proteins (lanes 4 and 5 in Figure 8). This result excludes the possibility that Band II is the D2 protein, which possesses one lysyl residue on its lumenal side. Since phosphorylated CP29 was reported to be located in this molecular mass region (*40*), we probed the blot with anti-phosphothreonine. However, we could detect little phosphorylated protein in this region, although we did find a small amount of phosphorylated D1 and D2 proteins (data not shown).

With increasing masking concentrations of NHS, the extent of subsequent NHS biotinylation after removal of Mn

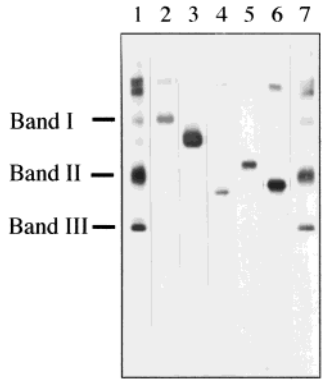


FIGURE 8: Identification of Bands I and II with antibodies against CP47, CP43, D1, D2, and LHC in $CaCl_2$−PS II core complexes that were masked with 50 mM NHS and further treated with 2 mM NH_2OH. Lanes 2−6 show immunoreactive bands against CP47, CP43, D1, D2, and LHC, respectively, which were detected by high-sensitivity luminescence (ECL). Lanes 1 and 7 show NHS-biotinylated proteins detected by avidin.

decreased for most proteins (lanes 4, 6, and 8 in Figure 7B), with the exception of Band I, the labeling of which remained relatively constant. We conclude, therefore, that biotinylation of the lysyl residue of CP47 is specifically dependent on the presence of functional Mn.

DISCUSSION

Which Process(es) of Photoactivation Is Modulated by Chemical Modification of Amine Groups? To explore the possible involvement of amino acids other than histidyl- and carboxyl-containing residues, we have studied effects of chemical modifiers of amine groups on photoactivation of the apo WOC. As shown in Figures 1 and 2, the chemical modifiers NHS, TNBS, and MAI caused a loss of capacity for photoligation of Mn^{2+} to the apo Mn complex. However, the compounds did not affect Mn^{2+}-dependent PS II donor activities. These results suggest direct involvement of amine groups on the PS II intrinsic protein(s) in photoligation of Mn^{2+} to the apo complex. Accordingly, on the basis of the currently recognized photoactivation mechanism described in the Results, chemical modification may affect the following: (1) photooxidation of Mn^{2+} at its binding sites, (2) the yield of L_2, a Mn^{3+}−Mn^{2+} intermediate generated during photoactivation, or (3) the yield of the dark reaction converting L_3 to an active Mn cluster.

Since chemical modification of amine groups did not affect DCIP photoreduction donated by Mn^{2+}, we can exclude the possibility that our treatment blocked the ability of PS II to photooxidize Mn^{2+}. In Figure 3, we found that the chemical modification caused a significant decrease in the number of high-affinity Mn-binding sites, but did not affect the low-affinity sites. These results indicate that amine groups of PS II protein(s) specifically function in setting the affinity of the high-affinity Mn-binding sites on the oxidizing side of PS II. We previously reported the same results obtained with PS II preparations chemically modified on histidyl residues (*18*) and carboxyl groups (*20*) of the D1 protein. We noted in Figure 1 that DCIP photoreduction donated by Mn^{2+} in chemically modified preparations was enhanced to some extent by the modification. This result seems to conflict with those in Figures 2 and 3. However, we also observed that chemical modification changed the high-affinity Mn-binding sites into low-affinity sites. Thus, we speculate that our treatments stimulated the exchange between Mn^{2+} or Mn^{3+} at the binding sites with those in the bulk phase, resulting in faster electron donation of Mn^{2+} to Yz^+.

To assess the intactness of the high-affinity Mn-binding sites, we used DCIP photoreduction supported by Mn^{2+} at sub-micromolar concentrations coupled with hydrogen peroxide (Figure 3). The K_m obtained with this method will be an apparent dissociation constant for dissociation of Mn^{2+} from its binding sites, assuming that the binding of Mn^{2+} to and dissociation of Mn^{2+} from its binding sites are much faster than electron donation from Mn^{2+} to Yz^+. Recently, Ono and Mino (*41*) showed through fluorescence decay kinetics that electron donation from Mn^{2+} to Yz^+ is biphasic, with 70% of the decay associated with the fast phase. In the fast component, the dissociation constant for dissociation of Mn^{2+} from its binding sites, K_d, was estimated to be 1.3 μM, with a stoichiometry of only one Mn^{2+} atom per PS II. In the study presented here, we found the fractions of Mn binding to depleted PS II complexes with different K_m values were approximately equal. When it is considered that PS II core complexes are the preparations obtained by further treatment of PS II membranes with detergents such as *n*-octyl β-D-glucopyranoside, K_{m1} and K_{m2} may reflect the fast and slow components observed by Ono and Mino (*42*), respectively. However, we cannot directly compare the different values for K_m and K_d, since they are close but not equivalent parameters.

To identify the steps of the photoactivation process that are affected by modulation of the high-affinity Mn-binding sites by NHS chemical modification, we studied the formation of the intermediates generated during photoactivation. In Figure 5, the rate constant for generation of L_2 was not changed by a 50% inhibitory NHS treatment, but that for decay of L_2 to D was significantly accelerated. The increase in this rate constant is probably the key to the decrease in yield of photoactivation, and suggests that the Mn^{2+}−Mn^{3+} binuclear dimer was destabilized by NHS. The same results were reported with thylakoid membranes from *Synechocystis* mutants lacking the entire *psb*O coding sequence (*42*) or in which the aspartic acids at positions 59 and 61 of the A−B lumenal loop of the D1 protein were mutated (*43*). In contrast, we previously reported that chemical modifications of histidine and acidic amino acid residues on the D1 protein lowered the rate constant for the decay of L_2, without any change in the rate constant for its generation (*19*). At present, it remains unclear why the mode of inhibition of the photoactivation process is dependent on the species of chemical modifiers, even though they all appear to damage the same high-affinity Mn-binding sites.

Identification of the Protein Chemically Modified by NHS in PS II. Of the PS II intrinsic proteins, D2, CP47, and CP43 possess lysyl residues located on the lumenal side of the membrane, while there are none on D1, Cyt b_{559}, PS II-I, or PS II-L. To identify a protein(s) involved in ligation of functional Mn, we examined the differences in NHS biotinylation between PS II preparations treated under various conditions (Figure 7). When PS II core complexes which had been depleted of all three extrinsic proteins but which retained Mn were masked with NHS, treated with NH_2OH to remove Mn, and then labeled with NHS-biotin, three proteins with molecular masses of 47, 33, and 22 kDa,

designated as Bands I−III, respectively, were significantly biotinylated. There was little labeling on CP43. Band I was identified as CP47 on the basis of its behavior in Western blots. Although Band II was initially considered to be the D2 protein from its position in gels, Western blots with the D1 and D2 antibodies excluded this possibility. Consideration of the molecular masses of the PS II intrinsic protein suggests that Band II is likely to be phosphorylated CP29, although we could not detect it with our antibody directed against phosphothreonine. On the basis of its position in gels, Band III is thought to be the 22 kDa PS II-S protein (*38*, *39*).

The large extrinsic loops, C and E, of CP47 on the lumenal side of PS II possess one and eight lysyl residues, respectively, and have been previously considered to play a role in water oxidation (*44*, *45*). Bricker's group reported that positively charged groups in the regions of ^{304}K−^{321}K and ^{389}K−^{419}K of loop E of CP47 can be chemically modified in the absence of the 33 kDa protein. On the basis of both site-directed mutagenesis (*34*, *35*) and their studies with the chemical modification reagents (*29*, *46*), they suggested that these residues are involved in the tight binding of the 33 kDa protein to the membranes. However, we suspect that the chemical modification site(s) on CP47 observed in this study may be different from the site for the 33 kDa protein binding, for the following reasons. (1) The rebinding of the 33 kDa protein was not affected when the PS II core complexes retaining the 33 kDa protein were treated with NHS (Figure 6), and (2) preparations which were masked with NHS and subsequently NHS biotinylated in the presence or absence of functional Mn displayed the same biotinylation pattern regardless of the presence of the 33 kDa protein (Figure 7 and data not shown). In addition, the fact that the CP47−D1−D2 subcore complex is simply isolated from thylakoid membranes with detergents (*47*, *48*) and the fact that the mutants lacking the whole *psbB* gene are not able to assemble PS II (*49*) both suggest that CP47 is closely associated with PS II reaction center proteins. Recently, Barber et al. (*50*) proposed a model in which the large E loop of CP47 interacts with the lumenal loops of the D2 protein. Studies with genetically engineered mutant cells (*26*) showed that deletion of ^{373}A−^{380}D or ^{384}R−^{392}V in the E loop of CP47 leads to a loss of the capacity for photoactivation. From these considerations, it is suggested that the amine group(s) located in the E loop function in photoligation of Mn^{2+} to the apo WOC, and that these are different from those involved in 33 kDa protein binding.

The sequences of CP29 and PS II-S, thought to be Bands II and III, respectively, have been shown to be highly homologous with those of the CAB gene products. They are located between PS II core proteins and LHC as peripheral intrinsic proteins, and have three and four transmembrane hydrophobic helices, respectively. Because of its capacity for binding violaxanthin (*51*) and sensing lumenal pH (*52*), CP29 is considered to regulate the chlorophyll *a* excited-state concentration of PS II. Also, recent studies on the three-dimensional structure of PS II with electron microscopy and single-particle image analysis (*53*) suggest that CP 29 may function in maintaining sequestered domains of inorganic cofactors required for oxygen evolution. Furthermore, Jegerschöld et al. (*54*) proposed that the sites clustering acidic amino residues in the CP29 lumenal loop specifically bind Ca^{2+} and are possibly involved in a pH-triggered structural change. Interestingly, Ca^{2+} is likely to stabilize the intermediates generated during the photoactivation process and enhance the yield of photoactivation; it must bind at its effector site so that stable photooxidation of a second Mn^{2+} can occur, resulting in formation of the second light-induced intermediate (*6*, *9*, *10*). On the other hand, PS II-S has been considered to be closely associated with the PS II extrinsic 33 and 23 kDa proteins, and may constitute a part of the WOC (*55*). This is in addition to its chaperonin-like function in the transient binding of pigments during biogenesis or turnover of chlorophyll-binding proteins (*56*).

CP29 and PS II-S have only two lysyl residues on the lumenal side, whereas they possess lysine-rich domains on the stromal side. As shown in Figure 7, the level of NHS biotinylation of Bands II and III decreased with increasing masking concentrations of NHS, while the level of biotinylation of Band I (CP47) remained almost constant. From these results, we suggest that NHS biotinylation observed with CP29 and PS II-S is due to insufficient masking by NHS of lysyl residues in the stromal loops, resulting from heterogeneous NHS modification caused by steric hindrance in the lysine-rich domains. At present, we cannot exclude the possibility that long-range or secondary effects associated with the removal of functional Mn cause an increase in the accessibility of NHS to other parts of CP29 or PS II-S (or even CP47). Recently, Büchel et al. (*57*) reported that the PS II core complexes mainly consisting of D1, D2, Cyt b_{559}, and CP47 can reconstitute a functional Mn cluster; however, its yield was low. This preparation appeared to contain neither CP29 nor PS II-S (*55*). Taken together, CP29 and PS II-S appear not to be essential for photoligation of Mn^{2+}, but may be required for the structural stability of PS II or proper structural positioning of the PS II extrinsic proteins. The lysyl residue(s) located on the lumenal side of CP47 is probably involved in photoactivation of the apo WOC, along with the histidyl and acidic amino acid residues of PS II reaction center proteins identified in previous studies.

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