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Iodination of D1 (herbicide-binding protein) is coupled with photooxidation of $^{125}I^-$ associated with Cl^--binding site in Photosystem-II water-oxidation system

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Specific iodination of D1 (the herbicide-binding protein) coupled with photooxidation of $^{125}I^-$ was found to occur not only in Mn-depleted Photosystem II (PS II) but also in Mn-retaining PS II membranes depleted of Cl^-. The following results pertain to the mechanism of iodination. (1) In Mn-retaining PS II, the extent of D1-specific iodination exhibited a parallelism with the capability of electron donation from I^- to PS II, suppressed completely by Cl^-, severely by F^- and CH_3COO^-, but not affected by SO_4^{2-}. In Mn-depleted PS II, however, the iodination was insensitive to Cl^-. (2) The affinity of I^- for PS II in supporting DCIP photoreduction was identical with the affinity of Cl^- in restoring O_2 evolution in Cl^--depleted PS II. (3) In Mn-retaining PS II, I^- not only diminished the height of thermoluminescence glow peak of Cl^--free abnormal S_2 state, but also reversed its abnormal peak position to normal position. (4) Repetitive flashes effected efficient iodination at shorter intervals, but not at a long interval of 10 s in both Mn-retaining and Mn-depleted PS II. These results were interpreted as follows. (i) In Mn-retaining PS II, I^- binds to the Cl^--binding site and is oxidized by Cl^--free abnormal S_2 to form a reactive species (probably I^+), which effects specific iodination of D1. (ii) In Mn-depleted PS II, I^- is oxidized by Z^+, the secondary donor of PS II, to form the reactive species to effect also specific iodination of D1. Assuming that the iodination site is close to the oxidation site of I^-, we propose that D1 protein bears a domain for Cl^--binding, S_2 (or Mn-binding) and Z-binding sites.

Introduction

The minimum unit for photosynthetic water oxidation so far isolated is a PS II reaction-center complex retaining Mn and the extrinsic 33 kDa protein [1,2]. This unit is composed of several proteins (CP47 apoprotein, CP43 apoprotein, D1, D2, 33 kDa extrinsic protein, cytochrome *b*-559 apoproteins and several low-molecular-weight proteins [3]) and cofactors (P-680, pheophytin, chlorophyll, Q_A, Q_B, Z, Mn and heme). Among these, the Q_B site has been proved to be located in a specific domain of D1 protein [4,5]. Based on structural and functional homology between plant PS II and bacterial reaction centers, it is assumed that P-680 and pheophytin are ligated with both D1 and D2, and Q_A is located in D2 [6,7]. In contrast, there is little information about the location of cofactors on the donor side of PS II, e.g., Z and Mn.

Iodine labeling of PS II protein(s) coupled with

Abbreviations: DCIP, 2,6-dichlorophenolindophenol; DPC, 1,5-diphenylcarbazide; Hepes, 4-(2-hydroxyethyl-1-piperazine-ethanesulfonic acid; LHC, light-harvesting complex; Mes, 4-morpholineethanesulfonic acid; PS II, Photosystem II.

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PS II photochemistry may give a clue to this problem. It is known that iodide anion (I^-) donates electron to PS II in NH_2OH-treated thylakoid [8] or reaction-center core complex [9], both of which are inactive in water oxidation but retain the secondary donor, Z. Recently using $^{125}I^-$ as an electron donor Takahashi and Asada [10] showed that a 29 kDa polypeptide in Tris-treated thylakoid is specifically labeled with ^{125}I under illumination. We demonstrated that the iodinated protein is D1 (herbicide-binding protein) both in Tris-treated PS II membranes of several higher plants and in Tris-treated reaction center complex of cyanobacterium [11]. In addition, Takahashi et al. [12] reported that D1 is photochemically iodinated in spinach PS II core complex depleted of the water-oxidation system. These results are interpreted as indicating that the site of electron donation from I^- to Z^+ is located close to D1.

Here we study the binding, electron donation and protein labeling capabilities of iodine-125 in Cl^--depleted water-oxidation system of various types of PS II membranes retaining Mn, and report that I^- associates with the Cl^--binding site in water oxidation system and donates electrons to the abnormal S_2 formed in the absence of Cl^-, and then finally labels D1.

Materials and Methods

O_2-evolving PS II membranes were isolated from wheat (*Triticum aestivum* L.) thylakoids by treatment with Triton X-100 according to Ref. 13 and stored in liquid N_2. After thawing, the PS II membranes were further treated with 1.2 M NaCl, 1.2 M $CaCl_2$ or 5 mM NH_2OH in 0.4 M sucrose and 40 mM Mes-NaOH (pH 6.5) for 30 min in the dark at 4° C (denoted as NaCl-PS II, $CaCl_2$-PS II and NH_2OH-PS II, respectively). Alternatively, the PS II membranes were treated in 0.8 M Tris-HCl (pH 9.0) for 30 min in the dark at 4° C (denoted as Tris-PS II). The treated membranes were collected by centrifugation at 30000 × *g* for 10 min, washed with 0.4 M sucrose and 40 mM Mes-NaOH (pH 6.5), then suspended in the same buffer but at pH 6.0. Almost complete depletion of Cl^- was attained by each of these washing procedures. Cl^- depletion of native PS II membranes was done by suspending in 0.4 M sucrose and 40 mM Hepes-NaOH (pH 7.5) according to Ref. 14 (denoted as Hepes-PS II). After 10 min incubation in the dark at 4° C, the membranes were precipitated and resuspended in 0.4 M sucrose and 40 mM Mes-NaOH (pH 6.0). Although Hepes-PS II, NaCl-PS II and $CaCl_2$-PS II membranes were devoid of some or all of the extrinsic proteins (18, 24 and 33 kDa), these membranes retained Mn and showed normal O_2 evolution when supplemented with Cl^-, Ca^{2+} and/or the extrinsic proteins [15]. In contrast, NH_2OH-PS II and Tris-PS II membranes were devoid of Mn and their water-oxidation activity could not be recovered without re-insertion of Mn by photoactivation process [16].

Iodination of these PS II membranes was done under continuous red light illumination for 15 min basically according to Ref. 11. Basic reaction medium was 40 mM Mes-NaOH (pH 6.0), 60 μM DCIP and 0.1 mM $Na^{125}I$ (0.1 mCi/ml, Amersham) containing 0.1 mg Chl/ml. Alternatively, PS II membranes supplemented with 1 mM 2,6-dichloro-*p*-benzoquinone instead of DCIP were illuminated with 99 xenon flashes (5 μs) at saturating intensity with varied dark intervals. After illumination, the labeled membranes were diluted with the same volume of 40 mM Mes-NaOH (pH 6.0) and 20 mM $MgCl_2$, collected by centrifugation at 13000 × *g* for 1 min at 25° C, washed once with the same buffer, and then subjected to SDS-polyacrylamide gel electrophoresis.

SDS-polyacrylamide gel electrophoresis was done on a separating gel containing 12.5% acrylamide, Neville's buffer [17] and 5.5 M urea as described in Ref. 2. Gels were stained with Coomassie brilliant blue R-250 and then subjected to autoradiography at −75° C using an intensifying screen and pre-flashed film.

DCIP photoreduction was measured spectrophotometrically with a Shimadzu UV300 spectrophotometer under saturating red light illumination from a 150 W xenon lamp. The PS II membranes (final concentration, 5 μg Chl/ml) were suspended in 0.4 M sucrose, 40 mM Mes-NaOH (pH 6.0) and 60 μM DCIP, unless otherwise stated. Molecular extinction of DCIP was assumed as 14.0 at 600 nm at pH 6.0 according to Ref. 18. In order to compare anion effect between O_2 evolu-

tion and DCIP reduction, O_2 evolution was measured polarographically at suboptimal pH 6.0 with a Clark-type electrode under saturating orange light illumination from a 650 W tungsten lamp according to Ref. 2. The PS II membranes (final concentration, 5 μg Chl/ml) were suspended in 0.4 M sucrose, 40 mM Mes-NaOH (pH 6.0) and 0.6 mM 2,6-dichloro-*p*-benzoquinone.

Thermoluminescence glow curves were measured as described in Ref. 19. The samples (300 μg Chl/ml) were dissolved in 40 mM Mes-NaOH (pH 6.0) or Hepes-NaOH (pH 7.5) and 0.4 M sucrose and an aliquot (80 μl) was applied to a sample holder, illuminated with a saturating xenon flash (5 μs) at 0°C, and then quickly frozen in liquid N_2. Alternatively, the samples were quickly mixed with NaI or NaCl (final concentration, 10 mM) immediately after flash excitation, incubated for 30 s at 0°C, and then frozen in liquid N_2.

Results

Chloride anion (Cl^-) is indispensable for water oxidation [20] and its removal reversibly inhibits S_2-to-S_3 transition, but not S_1-to-S_2 transition [21,22]. In Cl^--depleted NaCl-PS II membranes, Br^-, NO_3^- and I^- were partially substituted for Cl^- to restore O_2 evolution, while SO_4^{2-} had little effect (Table I). The observed efficiency order, $Cl^- > Br^- > NO_3^- > I^- > SO_4^{2-}$, was the same as already established for Cl^--depleted thylakoids [20] or PS II membranes [14,23]. The efficiency order observed in terms of DCIP photoreduction, however, exhibited a reversed order between I^- and NO_3^-: I^- restored the electron transfer from water to DCIP more effectively than NO_3^- did, nearly up to the level of Br^--supported rate, while I^- was less effective than NO_3^- in restoring O_2 evolution. This reversed efficiency order indicates that a significant part (at least 30%) of the I^--supported DCIP photoreduction was not coupled with O_2 evolution. It is of note that I^- does not affect the activity of other PS II reactions, e.g., DPC-supported DCIP photoreduction (not shown). Taking these data and the redox property of I^- reported in Ref. 8 into account, we may postulate that I^- is not only substituted for Cl^- in Cl^--depleted PS II to restore water oxidation but also donates electrons to the donor side of PS II in the absence of Cl^-.

Similar stimulation of DCIP photoreduction by I^- was observed in Cl^--depleted Hepes-PS II and $CaCl_2$-PS II membranes as well (Table II), although in this case I^- was less effective than NO_3^- in restoring O_2 evolution (not shown). The result indicates that the above-mentioned dual function of I^- is a common phenomenon for various types of Cl^--depleted PS II membranes retaining Mn. In contrast with these, only I^- but not Cl^- stimulated DCIP photoreduction in NH_2OH-PS II and Tris-PS II membranes which are devoid of Mn and incapable of O_2 evolution, but show a high DPC-supported activity (Table II) as already reported for NH_2-OH-treated thylakoids [8].

When $^{125}I^-$ was used to support DCIP photoreduction, specific iodination of D1 occurred in various types of PS II membrane (Fig. 1). Under

TABLE I

O_2 EVOLUTION AND DCIP PHOTOREDUCTION BY Cl^--DEPLETED NaCl-PS II MEMBRANES SUPPORTED BY VARIOUS ANIONS

Activity is expressed as μequiv·h^{-1} per mg Chl. The anions (10 mM) were added as sodium salt.

Addition	O_2 evolution	DCIP reduction
Cl^-	1088	334
Br^-	860	324
NO_3^-	328	204
I^-	268	292
SO_4^{2-}	204	66
None	232	62

TABLE II

DCIP PHOTOREDUCTION BY VARIOUS TYPES OF Cl^--DEPLETED PS II MEMBRANES SUPPORTED BY SALTS AND DPC

Activity is expressed as μequiv·h^{-1} per mg Chl. $CaCl_2$ (5 mM), NaCl (10 mM), NaI (10 mM) or diphenylcarbazide (0.5 mM) was added to support electron transfer.

Addition	PS II membrane				
	Hepes-II	NaCl-II	$CaCl_2$-II	NH_2OH-II	Tris-II
$CaCl_2$	–	386	136	22	20
NaCl	380	–	–	–	–
NaI	310	324	144	52	52
DPC	–	340	428	712	814
None	100	94	42	14	16

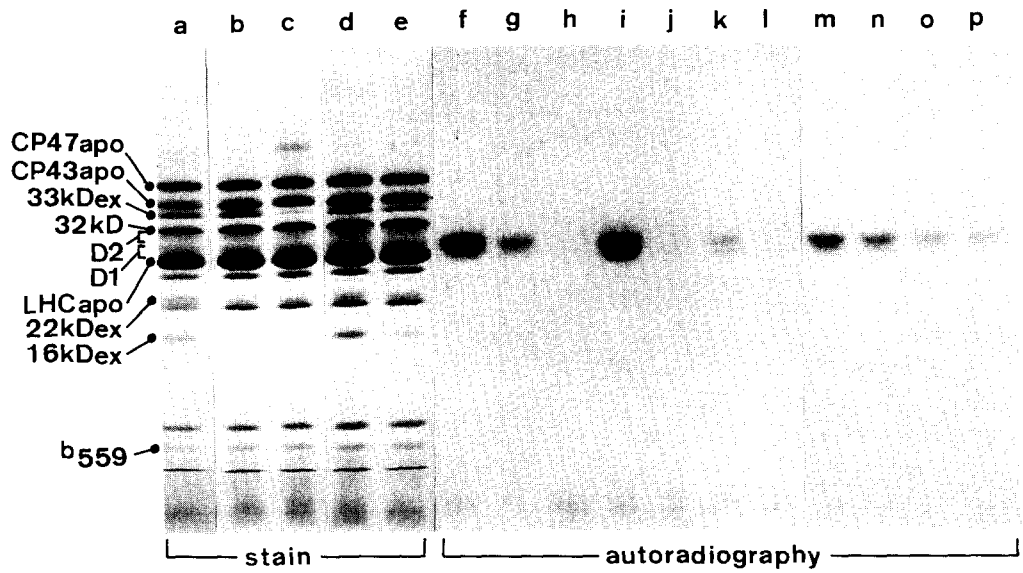

Fig. 1. Iodination of various PS II membranes by continuous illumination. Hepes-PS II (a, f, g, h), NaCl-PS II (b, i, j), $CaCl_2$-PS II (c, k, l), NH_2OH-PS II (d, m, n) and Tris-PS II membranes (e, o, p). The membranes were incubated with 100 mM Na_2SO_4 (f), 20 mM NaCl (h, j, l, n, p), or with no addition of anions (g, i, k, m, o).

illumination in the absence of Cl^-, D1 was labeled in all types of PS II membrane, while no labeling could be found in the dark (not shown). Presence of 20 mM Cl^- completely abolished the light-induced D1 iodination in Hepes-PS II, NaCl-PS II and $CaCl_2$-PS II membranes, while in contrast the iodination was not affected by Cl^- in NH_2OH-PS II and Tris-PS II membranes. The result implies that the Cl^--induced inhibition of D1 iodination is restricted to the PS II membranes retaining Mn, regardless of the presence or absence of extrinsic proteins. In view of the fact that the stable oxidant expected on the donor side of PS II is different in Mn-depleted and Mn-retaining membranes, Z^+ and S_2 or S_3 states, respectively, we may assume that the restrictive Cl^- inhibition arises from different properties of the two oxidants. Taking into account the fact that Mn-retaining PS II has a domain for Cl^--binding, the mechanism of the Cl^--induced inhibition may be either (a) Cl^- inhibits the association of I^- with Mn-retaining PS II membranes or (b) electrons supplied by Cl^--activated water oxidation competitively suppress the oxidation of I^-.

The intensity of D1 labeling in $CaCl_2$-PS II membranes was much weaker than that in NaCl-PS II membranes. This low labeling efficiency can be attributed to the low activity of DCIP photoreduction supported by I^- in $CaCl_2$-PS II membranes (Table II), and implies that Z^+ may not be the oxidant for I^- oxidation in Mn-retaining membranes, since Z^+ is expected to accumulate more readily in $CaCl_2$-PS II membranes than in NaCl-PS II membranes under continuous illumination as seen from the much lower water-oxidation activity in the former membranes. D1 labeling in Hepes-PS II membranes was also weaker than that in NaCl-PS II membranes, but was significantly stimulated up to the level of NaCl-PS II membranes by 100 mM SO_4^{2-} (Fig. 1, lane f). This can be interpreted as follows: the labeling of D1 in the Hepes-PS II membranes is partially suppressed by residual Cl^- strongly associated with PS II under acidic conditions, and excess SO_4^{2-} releases this suppression by dissociating the residual Cl^- from the membranes. It was separately confirmed that SO_4^{2-} does not inhibit the iodination so much (see Fig. 3).

The dependence of DCIP photoreduction on I^- concentration is shown in Fig. 2, NaCl-PS II membranes showed the highest affinity for I^- (apparent $0.2 < K_m < 0.5$ mM), while $CaCl_2$-PS II

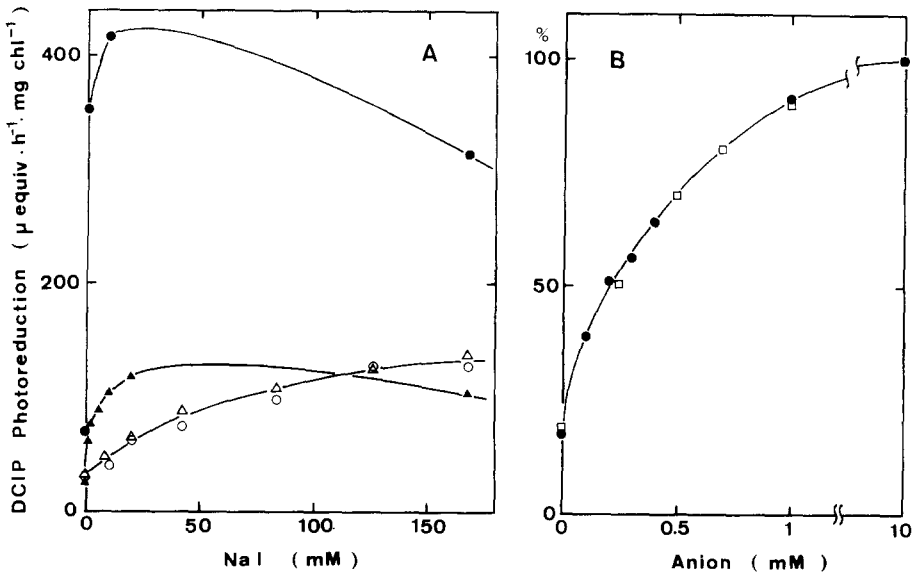

Fig. 2. Concentration dependency of NaI or NaCl on DCIP reduction of various types of PS II membrane. (A) ●, NaCl-PS II; ▲, $CaCl_2$-PS II; △, NH_2OH-PS II; ○, Tris-PS II. (B) I^--supported (●) and Cl^--supported (□) DCIP reduction by NaCl-PS II membranes. Note that I^--supported activity in (B) is the scale-expanded portion at low concentrations of NaCl-PS II curve in (A).

membranes showed a lower (apparent $K_m = 1.4$ mM) and NH_2OH-PS II and Tris-PS II membranes showed the lowest affinity (apparent $70 < K_m < 80$ mM). These dependencies are similar in their tendency to those reported for the affinity of Cl^- for water-oxidation system [24], although the apparent K_m values are considerably different. In Fig. 2B, the I^--dependence and Cl^--dependence of DCIP photoreduction by NaCl-PS II membranes are compared. Notably, these two dependence curves were almost identical with each other. These similarities in concentration dependence found between I^- and Cl^- suggest that the properties of I^--binding site are very similar to those of Cl^--binding site in water oxidation system.

To analyze more precisely the relationships between I^--binding site and Cl^--binding site, we investigated the competition between low concentration of Cl^- or I^- and high concentration of other anions (Table III). When applied alone, SO_4^{2-}, F^- and CH_3COO^- inhibited only slightly DCIP photoreduction. However, when these anions were added in combination with Cl^-, both F^- and CH_3COO^- severely suppressed the Cl^--induced stimulation, while SO_4^{2-} barely affected. Almost the same results were obtained for Br^--supported DCIP reduction. These results agree with the data measured in terms of O_2 evolution [25], and it can be interpreted that F^- and CH_3COO^-, but not SO_4^{2-}, are capable of displacing Cl^- by binding to the Cl^--binding site. This interpretation is consistent with the fact that F^- and CH_3COO^- have smaller ion volumes than Cl^-, while SO_4^{2-} has a larger ion volume than Cl^- [26]. Notably, the same anion specificity was found for I^--supported DCIP reduction (Table III). Al-

TABLE III

EFFECTS OF SO_4^{2-}, F^- AND CH_3COO^- ON Cl^-- OR I^--SUPPORTED DCIP PHOTOREDUCTION BY Cl^--DEPLETED NaCl-PS II MEMBRANES

Activity is expressed as $\mu equiv \cdot h^{-1}$ per mg Chl. NaCl (0.2 mM), NaBr (0.2 mM), NaI (0.2 mM) or diphenylcarbazide (0.5 mM) was added to support electron transfer. Anions (100 mM) were added as sodium salts to inhibit electron transfer.

Anion (100 mM)	Addition				
	none	DPC	NaCl	NaBr	NaI
None	80	396	232	248	204
SO_4^{2-}	62	312	164	196	162
F^-	52	306	52	58	56
CH_3COO^-	60	328	66	78	62

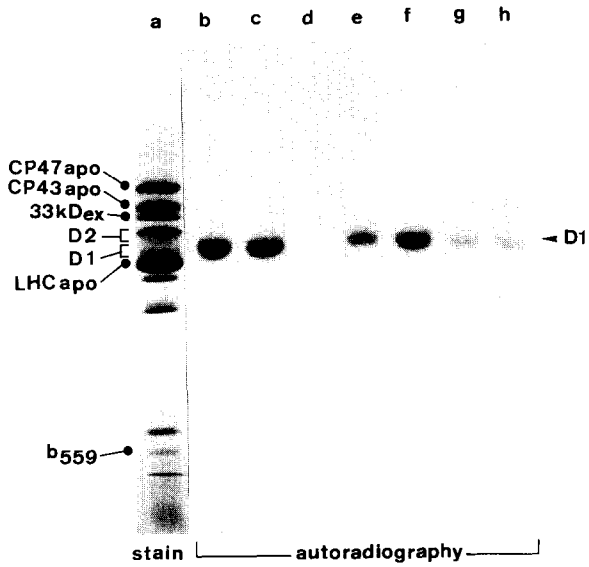

Fig. 3. Effect of excess anions, EDTA or DPC on D1 iodination in NaCl-PS II membrane. The membrane was photochemically iodinated with 0.1 mM $Na^{125}I$ in the presence of 0.5 mM EDTA (b), none (c), 20 mM NaCl (d), 100 mM NaF (e), 100 mM Na_2SO_4 (f), 100 mM $NaCH_3COO$ (g) or 0.5 mM DPC (h).

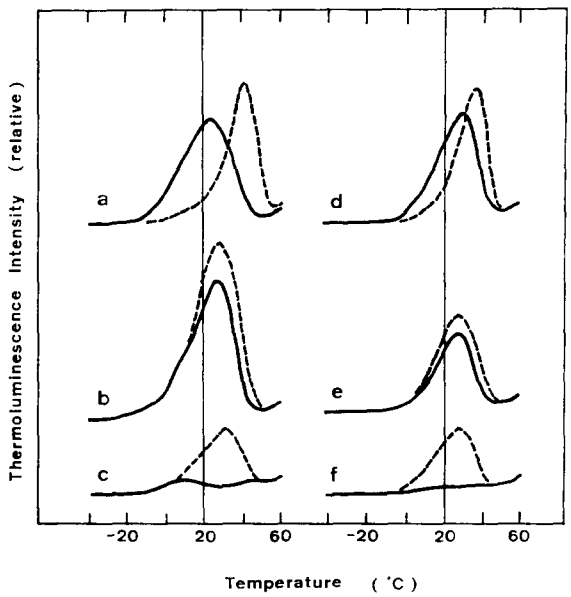

Fig. 4. Thermoluminescence glow curves of NaCl-PS II membrane. The membranes suspended in Mes buffer (pH 6.0) (solid line) or Hepes buffer (pH 7.5) (broken line) were flash-illuminated with no addition (a), 10 mM NaCl (b) or 10 mM NaI (c) and quickly frozen. Alternatively, they were illuminated without anions, incubated for 30 s with no addition (d), 10 mM NaCl (e) or 10 mM NaI (f) and frozen.

though we cannot separately quantify the electron flow from I^- and from water in this case, it is reasonable to assume that I^- binds to the Cl^--binding site in the Cl^--depleted water-oxidation system.

The same anion specificity was observed in terms of D1 iodination (Fig. 3). The iodination was completely abolished by Cl^- and severely suppressed by CH_3COO^- or F^-. In contrast, SO_4^{2-} only weakly affected the iodination. These results clearly indicate that not only accumulation of oxidant on PS II donor side by inhibition of O_2 evolution, but also a specific association of I^- with the Cl^--binding site is required for D1 iodination, and probably for oxidation of I^- by PS II as well. It was also shown that an efficient donor, DPC, which directly donates electrons to Z^+ and prevents S_2 accumulation, inhibited D1 iodination, and that extraction of loosely bound Ca^{2+} by EDTA did not affect the iodination. However, these results do not provide any conclusive information as to the oxidant species responsible for I^- oxidation, Z^+ or S_2.

The oxidant for I^- photooxidation in Mn-retaining PS II was investigated by means of thermoluminescence in NaCl-PS II membranes (Fig. 4). It is known that the thermoluminescence glow peak at around 25–35°C arises from charge recombination between S_2 and Q_B^- [19]. It is also known that in the absence of Cl^-, an abnormal S_2 (denoted as Σ_2) is formed, which shows a characteristic upshifted glow peak above 40°C [27]. The upshift is marked at neutral or slightly alkaline pHs, but not at acidic pHs below 6.5 (Ref. 23). Our NaCl-PS II membranes showed the typical glow peak due to $S_2Q_B^-$ recombination in the presence of Cl^- (Fig. 4b), but an abnormal glow peak due to $\Sigma_2Q_B^-$ recombination in the absence of Cl^- (Fig. 4a). The abnormally high peak temperature was marked at pH 7.5, but not at pH 6.0, in accordance with the previous report [23]. When I^- was present instead of Cl^-, the glow peak intensity was suppressed to be about half at pH 7.5 and practically abolished at pH 6.0 (Fig. 4c). Since the activity of I^--supported DCIP photoreduction was comparable to the Cl^--supported ac-

tivity (Tables II and III) and sensitive to diuron, a Q_B site inhibitor (not shown), accumulation of Q_B^- does not seem to be affected by I^-. Thus, the loss and suppression of the glow peak, at pH 6.0 and 7.5, respectively, is attributed to the loss of positive charge of the abnormal S_2 by electron donation from I^-.

Another information is available from the Fig. 4c glow curve. The glow peak in the presence of I^- at pH 7.5 was found at the normal peak position at around 30 °C. This implies that association of I^- to the Cl^--binding site resulted in conversion of Σ_2 to S_2 as reported for Cl^- [27]. This indication was further confirmed by post flash addition experiments shown in the right panel of Fig. 4, in which I^- or Cl^- was added to the membranes immediately after formation of Σ_2 by one flash illumination in the absence of Cl^-. Σ_2 has been reported to exhibit no EPR multiline signal [28] and to be converted in darkness to S_2 on addition of Cl^-[27]. Post addition of I^- abolished the glow peak at pH 6.0 and shifted the peak position back to the normal temperature at pH 7.5 with reduced intensity (Fig. 4f). The result clearly indicates that I^- efficiently donates an electron to Σ_2 at pH 6.0, and in addition converts Σ_2 to S_2 at pH 7.5 by substituting for Cl^- on association with the Cl^--binding site. It must be argued in this relation why the association of I^- with the Cl^--binding site at pH 7.5 does not result in the complete loss of the glow peak (broken curve f). Although we have not sufficient data at present, it is likely to presume that the capability of electron donation of I^- is significantly lower at pH 7.5 than at pH 6.0. This idea is consistent with our observation that the activity of I^--supported DCIP photoreduction at pH 7.5 is less than 25% of that at pH 6.0 (not shown). Apart from the limited electron donation capability at alkaline pHs, the data in Fig. 4 clearly indicate that the abnormal Cl^--free S_2 state oxidizes I^- which is associated with the Cl^--binding site in Mn-retaining PS II.

As a reference for the D1 specific photochemical iodination, we examined another iodination method, enzymatic iodination with lactoperoxidase and H_2O_2, which is known to iodinate the surface exposed tyrosine residues [29]. Lipids and most of PS II proteins were heavily labeled in all

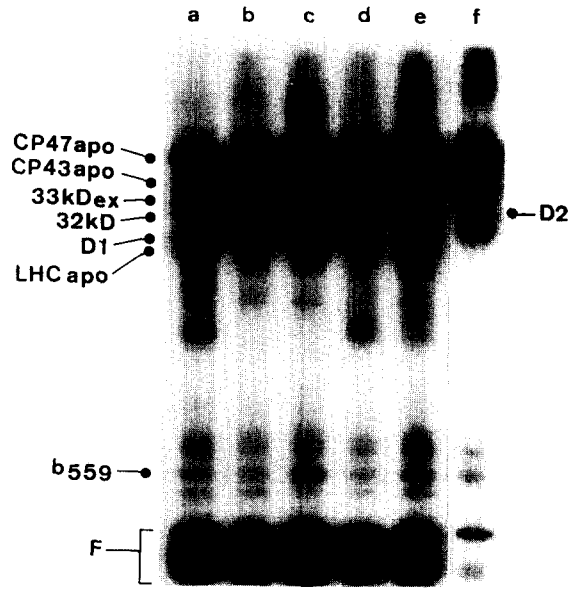

Fig. 5. Iodination of various types of PS II membrane and O_2-evolving reaction center core complex by lactoperoxidase and H_2O_2. Autoradiogram of non-treated PS II (a), NaCl-PS II (b), $CaCl_2$-PS II (c), NH_2OH-PS II (d), Tris-PS II membranes (e) and O_2-evolving PS II reaction-center core complex (f). F indicates free pigment region including lipids.

types of PS II (Fig. 5). Relating to the dye-staining intensity, CP47 apoprotein was found to be most heavily labeled, whereas the other PS II proteins were labeled roughly to the same extent with no specificity for D1. These broad specificities are in sharp contrast with the strict specificity (for D1) of photochemical iodination, and imply that the iodination sites on PS II proteins other than D1 are not labeled, in spite of their abundance, by photochemical iodination. Assuming that the lifetime of the reactive iodine species is fairly short, these differences in specificity may be interpreted as follows. In enzymatic iodination, all the surface-exposed tyrosine residues can be iodinated on access to lactoperoxidase, whereas in photochemical iodination the generation of reactive iodine species is restricted to the oxidation site; the Z-binding site in Mn-depleted PS II, while S_2 (or Mn-binding) site in Mn-retaining PS II, and binding of I^- to the Cl^--binding site will additively enhance the specificity and efficiency of D1 iodination.

A fairly strong labeling can be seen in D2 region (32 kDa). However, iodination of D2 ap-

pears unlikely because enzymatic iodination labeled D2 neither in O_2-evolving reaction center core complex at all (Fig. 5f) nor in non-O_2-evolving reaction center complex [12]. Probably this labeling spot is due to a comigrating 32 kDa protein which might originate from LHC [13]. Removal of the 33 kDa extrinsic protein by $CaCl_2$- or Tris-treatment enhanced the nonspecific iodination of the proteins including D1. This can be interpreted by the shielding effect of the 33 kDa extrinsic protein on PS II membrane proteins [30]. Effect of extraction of 18 kDa and 24 kDa extrinsic proteins by NaCl was hardly detected because these were removed from the non-treated membranes during incubation with lactoperoxidase and H_2O_2.

The mechanism of D1 iodination was investigated by flash-induced protein labeling. Specific labeling of D1 occurred under repetitive flash illumination as well as under continuous light both in Cl^--depleted NaCl-PS II and Tris-PS II membranes (Fig. 6). In both samples, flashes at shorter intervals gave rise to a higher extent of iodination, whereas those at a longer interval of 10 s gave only faint iodination. This indicates that at least two sequential photoreactions are required for protein iodination: I^- must be oxidized successively twice to generate a reactive species

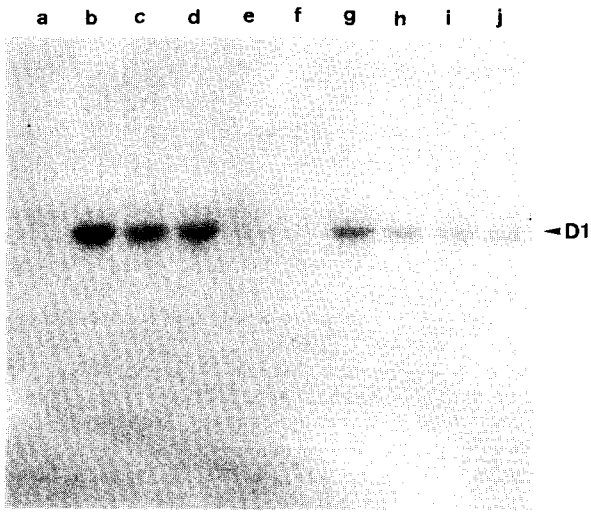

Fig. 6. Flash-induced iodination of NaCl-PS II and Tris-PS II membranes. The PS II membranes with $Na^{125}I$ were illuminated with 99 xenon flashes at intervals of 0.2 s (b, g), 0.5 s (c, h), 2 s (d, i) and 10 s (e, j) or only incubated in the dark (a, f).

(probably I^+) capable of protein iodination, and one electron-oxidized intermediate species decays or diffuses from the initial oxidation site during prolonged dark intervals. To effect D1 specific labeling, an iodide anion must possibly be anchored (or bound) in the vicinity of the oxidation site during the two equivalent oxidation reactions, since PS II generates only one oxidizing equivalent after each flash excitation as a form of Z^+ and Σ_2 (an abnormal S_2) in Tris-PS II (Mn-depleted) and Cl^--depleted NaCl-PS II (Mn-retaining) membranes, respectively. This scenario is consistent with the mechanism proposed for peroxidase activated protein iodination [31].

Discussion

The present study demonstrated that an efficient D1-specific iodination occurs not only in Mn-depleted PS II, but also in Mn-retaining PS II when depleted of Cl^-. Based on the observations presented in this paper, we propose that the D1-specific iodination in Mn-retaining PS II proceeds as follows: (i) specific binding of I^- to Cl^--binding site in the water-oxidation system depleted of Cl^-; (ii) generation of reactive iodine species (probably I^+) by sequential (at least two-step) photochemical oxidation of the bound I^- by use of the oxidizing equivalent accumulated as Σ_2 (an abnormal S_2); and (iii) modification by the reactive iodine species (probably I^+) of some amino acid residue(s) of D1.

As opposed to the oxidant (Z^+) assumed for photochemical D1 iodination in Mn-depleted PS II [11,12], we assumed Σ_2 as an oxidant in the above scheme. This assumption is based on the observation that Σ_2 is the most probable and stable oxidizing equivalent generated by one flash excitation of Cl^--depleted PS II [27], and that Σ_2 is readily eliminated by post-flash addition of I^- (Fig. 4). Although these observations are not always unambiguous evidence, the view is very likely in reference to the fact that Cl^- is involved in maintaining the structure of S_2, as evidenced by reversible disappearance and appearance of the S_2 multiline EPR signal on post-flash Cl^--depletion and Cl^--repletion [28]. The assumption of I^- binding to the Cl^--binding site may conveniently

account for the efficiency and specificity of photochemical iodination in Mn-retaining PS II. Since at least two equivalents of I^- oxidation are needed to generate a reactive iodine species, I^- must be localized in its initial oxidation site at least during the flash interval. The Cl^--binding site would conveniently provide an I^- anion with these conditions. Thus, assuming that the lifetime of reactive iodine species (probably I^+) is fairly short, and the iodination site and I^- oxidation site are the same or very close to each other, we may conclude that both sites for Cl^--binding and S_2 (or Mn-binding) are located on D1 protein rather than on other PS II proteins.

In Mn-depleted PS II, the oxidant for I^- oxidation may be a priori assumed to be Z^+, which is the sole light-inducible stable oxidant. In spite of long life time of Z^+ reported for Tris-washed thylakoids, however, the iodination extent in Mn-depleted PS II was far lower than that in NaCl-treated (Mn-retaining) PS II. This is probably because Mn-depleted PS II has a low affinity for I^- (Fig. 2) and also low efficiency in I^- oxidation (Table II). In other words, the efficiency of iodination does not simply depend on the lifetime of Z^+, but on the rate of I^- oxidation. It may be of note in this relation that the iodination efficiency in Mn-depleted PS II is relatively higher under flash illumination (Fig. 6) as compared with under continuous illumination (Fig. 1). It may be also of note that D1-specific iodination occurred even in the absence of Cl^--binding site, since the iodination in Mn-depleted PS II was totally resistant to Cl^-. At present we have no reliable experimental data as to the mechanism for this specificity, but the following speculations: the exchangeability of iodine at the oxidation site is limited due to (i) the low mobility of one equivalent oxidized iodine species, or (ii) the structural properties of the Z-binding site. The former, if it is the case, will enhance the iodination efficiency and specificity not only in Mn-depleted but also in Mn-retaining PS II. The latter assumes a pocket for Z-binding site in reference to the proposal that Z is burried 1.3–1.4 nm deep from the lumenal surface of thylakoids [32]. Apart from these details as to the mechanism for localizing I^-, the D1 specific iodination in Mn-depleted PS II suggests that Z^+ is located on D1 as well as the Cl^--binding site and S_2 (or Mn-binding site) are.

It is generally considered that enzymatically activated protein iodination occurs mainly in tyrosine residues on the surface of proteins [33]. If we assume the same for photochemical iodination of D1, several tyrosine residues (Tyr 73, 94, 107, 112 and 161) are pointed out on the proposed membrane-spanning model of D1 (Refs. 7 and 30) as candidate residues to be modified. According to our preliminary results of enzymatic digestion of iodinated D1, the iodinated residues are found between the N-terminal and the amino acid around arginine 225, and all the above candidate residues are contained in this region. It is not clarified yet whether one or some specific or all the tyrosine residues among the above candidates are iodinated, and whether the same or different residues are iodinated in Mn-retaining and Mn-depleted PS II. Determination of the iodinated residue will no doubt provide more precise information about the location of Z^+, S_2 (or Mn-binding site) and the Cl^--binding site.

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