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Specific ^{125}I labeling of D1 (herbicide-binding protein)

An indication that D1 functions on both the donor and acceptor sides of photosystem II

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When $^{125}I^-$ was given as an artificial electron donor to non-O_2-evolving thylakoids of spinach, a 29 kDa polypeptide was specifically tagged by ^{125}I due to its photooxidation by PS II [(1985) Plant Cell Physiol. 26, 1093–1100]. We examined precisely the ^{125}I-labeling pattern in comparison with azido[^{14}C]atrazine photoaffinity labeling of D1 and immunoblotting with anti-D1 and anti-D2, and found that D1 (herbicide-binding protein) of PS II reaction center complex is specifically tagged by ^{125}I in three different species of higher plants (spinach, pea and wheat) and a thermophilic cyanobacterium *(Synechococcus vulcanus)*. It was suggested that D1 bears the photooxidation site or has a domain very close to the photooxidation site on the donor side of PS II, in addition to the well established binding site for Q_B and herbicides on the acceptor side of PS II.

Iodination; Photosystem II; Protein D1; Herbicide-binding protein; Photosynthesis

1. INTRODUCTION

The donor side of PS II is known to involve at least two redox components other than the reaction center chlorophyll (P-680): the secondary donor, Z, and Mn of the water oxidase system. Although recent protein composition analyses of non-O_2-evolving [1,2] and O_2-evolving [3–5] PS II reaction center complexes have revealed that the complex is composed of five or six major proteins, CP47 apoprotein, CP43 apoprotein, D1, D2 and cytochrome *b*-559 (and the extrinsic 33 kDa protein in the latter complex), none of these proteins have so far been identified as Mn-binding or Z-binding protein.

A unique method for attacking this problem would be ^{125}I labeling reported by Takahashi and Asada [6]: $^{125}I^-$ given to Tris-treated spinach thylakoids specifically labels a 29 kDa protein upon illumination. Since the labeling of this protein is observed only under the conditions where I^- donates electrons to the secondary donor of PS II [7], they proposed that this protein bears the secondary donor or is located in the vicinity of the secondary donor on some other protein(s). Based on the mobility in SDS-PAGE and resistance to extraction with a chaotropic reagent, this also suggested that the tagged 29 kDa protein was not D1 but some other integral protein of PS II, possibly D2.

In this study we purified the reaction center complex from ^{125}I-labeled PS II membranes, and compared the ^{125}I-labeling pattern with the patterns of azido[^{14}C]atrazine photoaffinity labeling

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Abbreviations: Mes, 2-(*N*-morpholino)ethanesulfonic acid; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; PS II, photosystem II

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and immunoblotting with anti-D1 and anti-D2. It was unambiguously demonstrated that D1 is mainly tagged by ^{125}I in all types of PS II preparations obtained from pea, spinach, wheat and a thermophilic cyanobacterium, *Synechococcus vulcanus*.

2. MATERIALS AND METHODS

O_2-evolving PS II membranes were isolated from spinach (*Spinacia oleracea* L.), pea (*Pisum sativum* L.) and wheat (*Triticum aestivum* L.) by treatment of their thylakoids with Triton X-100 according to [8,9]. O_2-evolving particles of the thermophilic cyanobacterium, *Synechococcus vulcanus*, enriched in PS II reaction centers, were isolated by treatment of its thylakoids with lauryldimethylamine-*N*-oxide according to Koike and Inoue [10].

These PS II preparations were treated with 0.8 M Tris-HCl (pH 9.0) for 30 min at 4°C, collected by centrifugation, resuspended in 50 mM Hepes-NaOH (pH 7.5) and 10 mM NaCl, and then subjected to ^{125}I labeling basically according to [6]. Tris-treated PS II samples were dissolved (0.1 mg Chl/ml) in 0.1 mM $Na^{125}I$ (0.1 mCi/ml, Amersham), 60 μM 2,6-dichlorophenol indophenol, 10 mM NaCl and 50 mM Hepes-NaOH (pH 7.5), and illuminated in a 1 mm thick cell with a fluorescent lamp through a red filter (>600 nm, 0.3 mW/cm^2) for 15 min at 25°C. The reacted samples of higher plants and the cyanobacterium were collected by centrifugation at 13000 × *g* for 1 min at 25°C and at 41000 × *g* for 30 min at 4°C, respectively, preceded by a 10-fold dilution with 50 mM cold Hepes-NaOH (pH 7.5) and 10 mM NaCl in the latter case.

For higher plant samples, the respective reaction center complexes were isolated from the ^{125}I-labeled PS II membranes basically as described [9]. The labeled samples were suspended in 40 mM β-octylglucopyranoside, 20 mM Mes-NaOH (pH 6.0), 10 mM NaCl, 5 mM ϵ-aminocaproic acid and 1 mM benzamidine to give a final concentration of 0.6–0.9 mg Chl/ml, and centrifuged at 41000 × *g* for 30 min at 4°C. An aliquot (0.5 ml) of the supernatant containing solubilized reaction center complex was layered onto a discontinuous sucrose density gradient (0.5 ml of 0.3 M and 0.5 ml of 0.9 M sucrose solutions containing 40 mM β-octylglucopyranoside, 20 mM Mes-NaOH (pH 6.0), 10 mM NaCl, 5 mM $CaCl_2$, 5 mM ϵ-aminocaproic acid and 1 mM benzamidine), and centrifuged on a Hitachi RP83T angle rotor at 165000 × *g* for 6 h to pellet the reaction center complex.

O_2-evolving higher plant PS II membranes and cyanobacterial PS II reaction center particles were labeled with azido[^{14}C]atrazine according to [11]. For higher plant samples, the labeled PS II reaction center complex was isolated as described above except that a higher β-octylglucopyranoside concentration (50 mM) was used.

Rabbit antisera against D1 and D2 proteins were prepared by using electrophoretically purified spinach D1 and D2 as antigens. Electroblotting and immunodetection were done following standard methods [12] using a Biorad immunoblot kit with a nitrocellulose membrane and peroxidase-conjugated goat anti-rabbit IgG.

SDS-PAGE was performed on a composite separating gel (12.5% and 14.5% acrylamide in the upper two-thirds and lower one-third, respectively) containing 5.5 M urea as described in [5] unless otherwise stated. Gels were stained with Coomassie brilliant blue R-250, and then subjected to autoradiography (for ^{125}I) and fluorography (for ^{14}C).

3. RESULTS AND DISCUSSION

The O_2-evolving reaction center complexes from three different species of higher plants showed very similar dye-stained protein patterns composed of six major bands (fig.1A). Based on [2,5,13], these components are assigned as CP47 apoprotein, CP43 apoprotein, 33 kDa extrinsic protein, D2, D1 and cytochrome *b*-559 apoprotein. This assignment of D1 was verified by photoaffinity labeling with azido[^{14}C]atrazine. As shown by the fluorograms in fig.1A, only one labeled band from each of the three PS II membranes could be seen, indicative of specific labeling of the D1 protein [11]. The labeled protein was exclusively recovered in the reaction center complex, and its band position in the fluorogram exactly corresponded to the dye-stained diffuse bands indicated as D1. As a result of this assignment of the D1 band, another diffuse band found between D1 and the 33 kDa extrinsic protein was in turn assigned to D2. By

almost the same procedures, D1 and D2 in cyanobacterial O_2-evolving PS II particles were assigned (fig.1B).

The above assignment of D1 and D2 was further confirmed by immunoblotting. Our antisera prepared against spinach D1 and D2 specifically crossreacted with authentic D1 and D2 of *Chlamydomonas reinhardtii* PS II particles, respectively (not shown). As fig.2 shows, these antisera reacted not only with D1 and D2 of spinach and two other higher plants but also with those of *Synechococcus vulcanus*, although the crossreaction with cyanobacterial D1 was appreciably weaker than with others. The fact that cyanobacterial D1 and D2 crossreact with anti-D1 and anti-D2 of higher plants, respectively, suggests highly conserved structures of these two proteins among higher plants and cyanobacteria. It is of note in this context that the anti-D2 recognized D2 as doublets in all the three higher plants but as a singlet in the cyanobacterium. This is probably due to phosphorylation in an appreciable subpopulation of D2 in higher plants [14] but none or less in cyanobacteria. These results are completely consistent with the assignments in fig.1 and unambiguously justify our identification of D1 and D2.

^{125}I was incorporated into Tris-treated PS II membranes of higher plants and cyanobacterial PS II reaction center complex, and for higher plant samples the reaction center complexes were isolated from the ^{125}I-labeled membranes. As shown in fig.3, upper panel, the protein composition of the reaction center complexes thus prepared was almost identical with that of the O_2-evolving reaction center complexes shown in fig.1, except for the absence of the 33 kDa extrinsic protein due to its removal by Tris treatment. The fluorograms of these gels (lower panel) indicate that ^{125}I

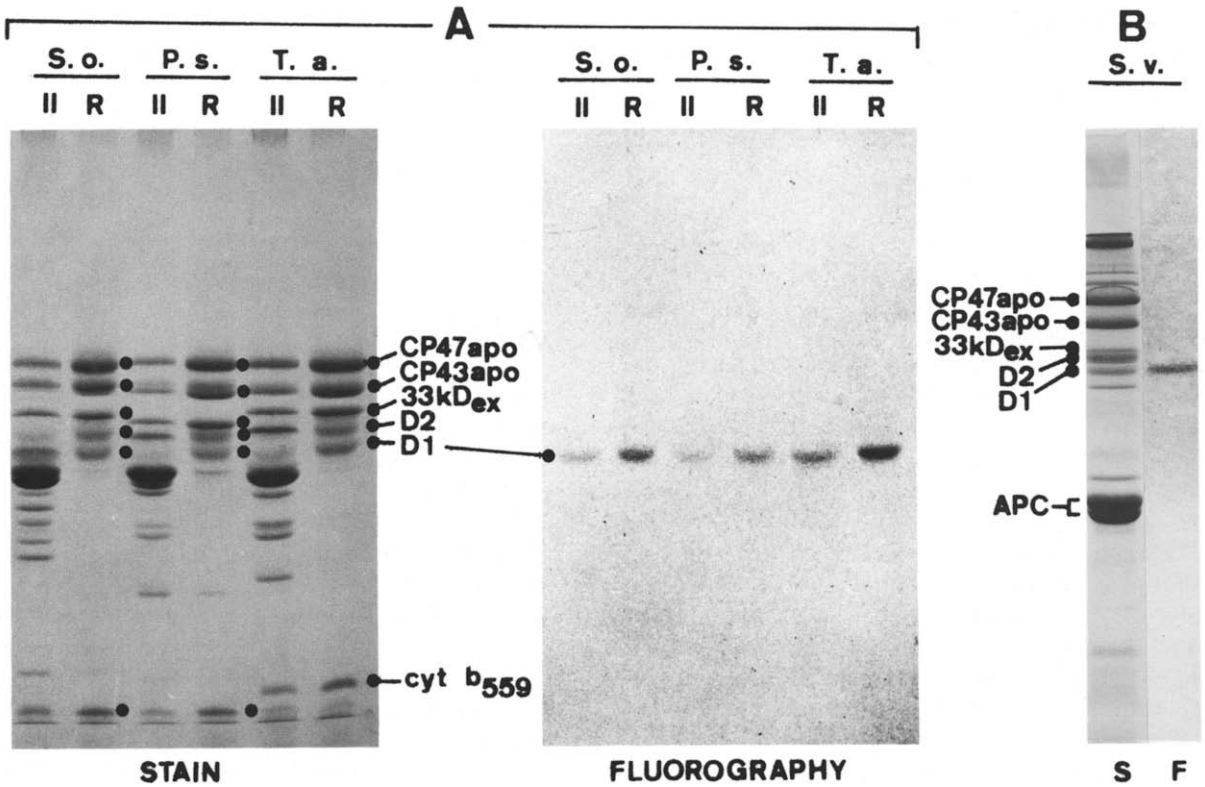

Fig.1. Azido[^{14}C]atrazine photoaffinity labeling of the PS II components. (A) Dye-stained protein profiles (left panel) and their fluorograms (right panel) of PS II membranes (II) and O_2-evolving reaction center complexes (R) of spinach (S.o.), pea (P.s.) and wheat (T.a.). The reaction center complexes were isolated from the labeled membranes. (B) Dye-stained protein profile (S) and its fluorogram (F) of O_2-evolving PS II reaction center particles of *Synechococcus vulcanus* (S.v.). A urea-containing 14.5% acrylamide gel was used for this sample. APC, allophycocyanin.

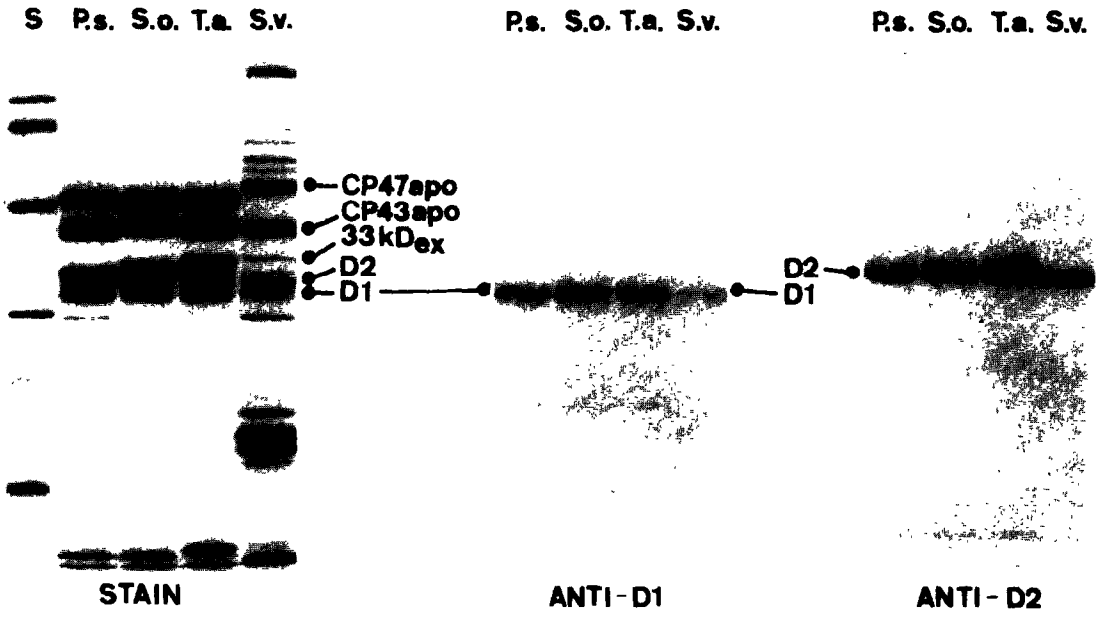

Fig.2. Immunoblotting detection of D1 and D2 in PS II reaction center complexes. (Left) Dye-stained protein profiles; (middle) immunodetection with anti-D1; (right) immunodetection with anti-D2. P.s., pea; S.o., spinach; T.a., wheat; S.v., *Synechococcus vulcanus*.

preferentially labeled D1 in both the three higher plants and cyanobacterium. In addition to D1, several diffuse bands in the high-molecular-mass region (>60 kDa) were clearly but more weakly labeled in higher plant samples. These bands were exclusively recovered in the reaction center core complexes after fractionation, indicative of specific association of these components with the reaction center. Since no such high-molecular-mass components could be found in the dye-stained profiles of non-Tris-treated PS II membranes, it is likely that they are the products of aggregation, due to Tris treatment or iodination, of the reaction center polypeptides including the labeled D1 protein. In pea and wheat CP47 apoprotein and D2 were also weakly but significantly labeled, while they were not in spinach. In cyanobacterium, no label was found in the high-molecular-mass region nor in CP47 apoprotein, while D2 was weakly labeled.

It has been established that ^{125}I labeling of thylakoid proteins occurs only when I^- donates electrons to the secondary donor of PS II [6,7]. The labeling requires light illumination and is inhibited by diuron, an inhibitor of PS II electron transfer, or suppressed by diphenylcarbazaide, an electron donor alternative to I^-. In untreated thylakoids with active oxygen evolution, no labeling occurs. Based on these characteristics, Takahashi and Asada [6] concluded that photooxidation of I^- at the donor side of PS II results in ^{125}I labeling, although the exact site of I^- photooxidation has not yet been identified. If we interpret our data, the specific ^{125}I labeling of D1, based on their conclusion, we can assume that D1 has a role on the donor side of PS II in addition to its well established role on the acceptor side, Q_B and herbicide binding.

Photooxidation of I^- by the secondary donor of PS II is supposed to produce either radical ($I^\cdot$) or iodinium (I^+). Both species are highly reactive with tyrosine residues in the vicinity of the secondary donor [6,15]. Tyrosine residues are abundant not only in all the reaction center components but also in the apoproteins of light-harvesting chlorophyll *a/b*-protein complex II: 17, 15, 8 and 12 tyrosine residues are coded for spinach CP47 apoprotein, CP43 apoprotein, D2 and D1, respectively [16–18]. In spite of such abundant attacking sites thus expected, only the D1 protein among others

was heavily and specifically labeled by ^{125}I on illumination in all the samples tested. This implies that ^{125}I labeling occurs in a very restricted domain near the photooxidation site, which is probably located on D1 protein.

In view of the above proposed dual function of D1 on both the acceptor and donor sides of PS II, our data also seem to be related to the recent observation on the LF-1 mutant of *Scenedesmus*, in which a mutation on D1 processing affects the PS II donor side: newly synthesized D1 with transit peptides is not processed but accumulates in the mutant thylakoids [19,20] and prevents Mn ligation possible by steric hindrance due to the extra peptides or by the failure of full integration of D1 into the reaction center complex. It may also be speculated that our data are relevant to the proposed view that D1 and D2 of higher plants are homologous to L and M subunits of the reaction center of photosynthetic purple bacteria, respectively, especially in the regions for binding of bacteriochlorophyll, bacteriopheophytin, Q_A and Q_B [21–33]. In fact, Satoh [24] isolated a D1/D2/cytochrome *b*-559 complex as a minimal photochemical unit of higher plant PS II. According to the crystallographic analysis [25,26], the bacterial (*Rhodopseudomonas viridis*) L and M subunits ligate bacteriochlorophyll *b* (primary donor) and quinones (secondary acceptor), and also make contact with *c*-type cytochrome (secondary donor): both subunits cooperatively function on the donor side as well as on the acceptor side. Our results about D1 in this paper are in accordance with this view, although we have no such information about D2 at present.

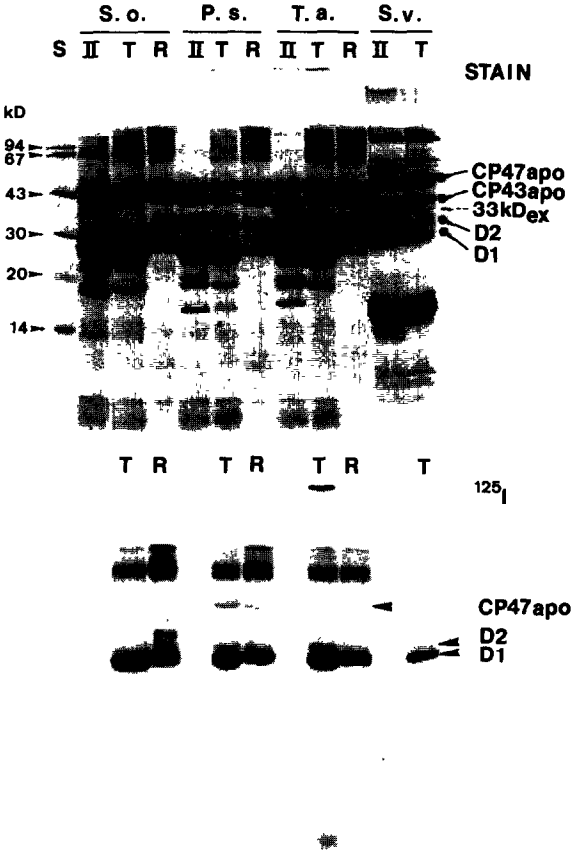

Fig.3. ^{125}I labeling of the PS II components of spinach (S.o.), pea (P.s.), wheat (T.a.) and a cyanobacterium, *Synechococcus* (S.v.). (Upper panel) Dye-stained protein composition of the labeled PS II reaction center complexes (R) isolated from Tris-treated and then ^{125}I-labeled PS II membranes (T). (Lower panel) Autoradiograms of the upper panel gels. In the upper panel, non-Tris-treated (and non-labeled) samples (denoted as II) were also analyzed to show complete extraction by Tris treatment of the three extrinsic proteins including the 33 kDa protein (denoted as 33 kD_{ex}).

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