Evidence for a dual function of the herbicide-binding D1 protein in photosystem II

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The LF-1 mutant of the green alga *Scenedesmus obliquus* is completely blocked on the oxidizing (water-splitting) side of photosystem II (PS II) while the reaction center and reducing side remain functional. A 34-kDa protein found in the PS II reaction center core complex of wild-type cells is replaced by a 36-kDa protein in the mutant cells. Both of these proteins are labeled by azido[^{14}C]atrazine and are recognized by polyclonal antibodies raised against the herbicide-binding, D1 protein of *Amaranthus hybridus*. The data provide a new perspective on the role of the D1 protein by implying that it affects the oxidizing side of PS II in addition to performing its well established function on the reducing side.

Azidoatrazine *Herbicide binding* *Photosystem II* *Oxygen evolution* (Scenedesmus, *LF-1 mutant*)

1. INTRODUCTION

The photochemical and electron-transfer reactions of PS II drive the reduction of plastoquinone on one side of the thylakoid membrane and the oxidation of water on the other [1]. Current research is attempting to define the polypeptide composition of the PS II complex, with special emphasis on the specific role of individual proteins (and protein domains). These studies are being influenced strongly by the recently derived X-ray crystal structure of the reaction center from the purple photosynthetic bacterium, *Rhodopseudomonas viridis* [2]. However, in spite of certain homologies between the bacterial reaction center proteins and those from PS II reaction center core complexes [2–4], the assignment of most PS II electron-transfer components to specific proteins has been difficult.

An exception to this uncertainty has been the localization of the binding site for triazine herbicides, such as DCMU and atrazine, which block electron transfer between the primary and secondary acceptors (Q_A and Q_B, respectively) on the reducing side of PS II. Analyses of herbicide-resistant mutants have revealed that the protein product of the chloroplast-encoded *psbA* gene is directly involved in binding these inhibitors, and by inference in binding Q_B [5,6]. Here, we will refer to the *psbA* gene product as the D1 protein [7]. It has also been called the Q_B-, the 32 kDa, and the herbicide-binding protein (review [8]). A photoaffinity labeling technique employing azido[^{14}C]atrazine, an analog of atrazine, has been used to identify the D1 protein after separation of

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Abbreviations: azidoatrazine, 2-azido-4-ethylamino-6-isopropylamino-*s*-triazine; Chl, chlorophyll; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; LDS, lithium dodecyl sulfate; LF, low fluorescent; PAGE, polyacrylamide gel electrophoresis; PS, photosystem; Q_A, primary PS II quinone acceptor; Q_B, secondary PS II quinone acceptor; WT, wild type

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thylakoid proteins by anionic detergent (SDS or LDS) PAGE [9]. Estimates of the molecular mass of the labeled protein, based on its mobility, range between 30 and 34 kDa [8,9].

In an independent line of research, a 34-kDa polypeptide component of the PS II core complex, has been implicated as having a function on the oxidizing side of PS II. Identification of this protein has relied on analyses of LF mutants of the green alga *Scenedesmus obliquus*. Several of these mutants have an identical phenotype [10], including loss of water-oxidation capability, loss of ~60% of thylakoid-bound Mn, and relatively unimpaired PS II reaction center and reducing side activity [10–12]. These mutants show loss of the 34-kDa protein and the appearance of a 36-kDa protein not present in the WT membranes. Recently, we demonstrated that the azido[^{14}C]atrazine photoaffinity technique labels both the 34-kDa protein in the WT algae and the 36-kDa protein in the LF-1 mutant [13]. Since electron transport properties on the reducing and oxidizing sides of PS II traditionally have been assigned to distinctly different proteins, we have investigated the relationship of the azido[^{14}C]atrazine-labeled proteins in *Scenedesmus* to those studied in higher plants.

2. MATERIALS AND METHODS

WT *S. obliquus* and the LF-1 mutant were grown heterotrophically in the dark on enriched medium [14]. Cells were disrupted using glass beads and thylakoid membranes collected by differential centrifugation [12]. Spinach was purchased from a local market and thylakoids isolated as described in [9]. Membranes were labeled with azido[^{14}C]atrazine (49 mCi/mmol, Pathfinders Laboratories, St. Louis, MO) using the buffers and protocol of [9]. Samples, at 50 μg Chl/ml, were made to 1 μM azido[^{14}C]atrazine and irradiated with short-wavelength UV light (model UVSL-15, Ultra Violet Products, San Gabriel, CA) for 30 min at 4°C, with stirring. Labeled thylakoids were collected by centrifugation and suspended in 10 mM Tricine, pH 7.8, 10% sucrose at 1 mg Chl/ml. Samples were incubated for 45 min at 30°C either without or with a lysine-specific protease (endoproteinase Lys-C, Boehringer-Mannheim) at 0.005 U/μg Chl [15]. These samples were then made to 1% LDS and 50 mM dithioerythreitol, and their proteins separated by LDS-PAGE using 10–15% acrylamide gradients [13]. After electrophoresis the gel was stained (Coomassie brilliant blue), photographed, and prepared for fluorography [16] using EN^{3}HANCE (New England Nuclear). Fluorography was for 3 weeks at −75°C using Kodak X-Omat AR film.

PS II core particles were isolated from PS II-enriched membranes [12,17] by solubilization with 1% dodecyl-β-D-maltoside and centrifugation on sucrose density gradients [18]. The PS II core-containing fractions were prepared for LDS-PAGE as described above except that the samples were heated (60°C for 4 min) prior to electrophoresis. Two sets of samples were separated on one gel. Half of the gel was stained with Coomassie brilliant blue, and the proteins in the other half were transferred to nitrocellulose paper [19]. The blot was probed with rabbit polyclonal antibodies raised against a 22-kDa fragment of the D1 protein of *Amaranthus hybridus* [20]. The bound antibodies were localized using a commercial assay kit (Bio-Rad Laboratories) which employs horseradish peroxidase conjugated to goat antibodies against the F_c portion of rabbit antibodies.

3. RESULTS

Fig.1A (lanes 1–3) shows the polypeptide profiles obtained after LDS-PAGE of azido[^{14}C]-atrazine-labeled thylakoids from spinach, WT *Scenedesmus* and the LF-1 mutant. Although there are many proteins present in these thylakoid samples, examination of the fluorograph obtained from this gel (fig.1B, lanes 1–3) reveals that in each thylakoid sample a single radioactive band is detected. Proteins with equivalent mobilities, indicating a molecular mass of 34 kDa, are labeled in spinach and WT *Scenedesmus*, while a 36-kDa band is labeled in the mutant.

The amino acid sequence of the spinach D1 protein has been deduced from the nucleotide sequence of its *psbA* gene [21]. One of the features of the spinach protein, as well as the D1 protein from several other species [22], is that it lacks lysine residues. In contrast, very little is known about the *Scenedesmus* chloroplast genome, and its *psbA* gene has not been characterized. Labeled

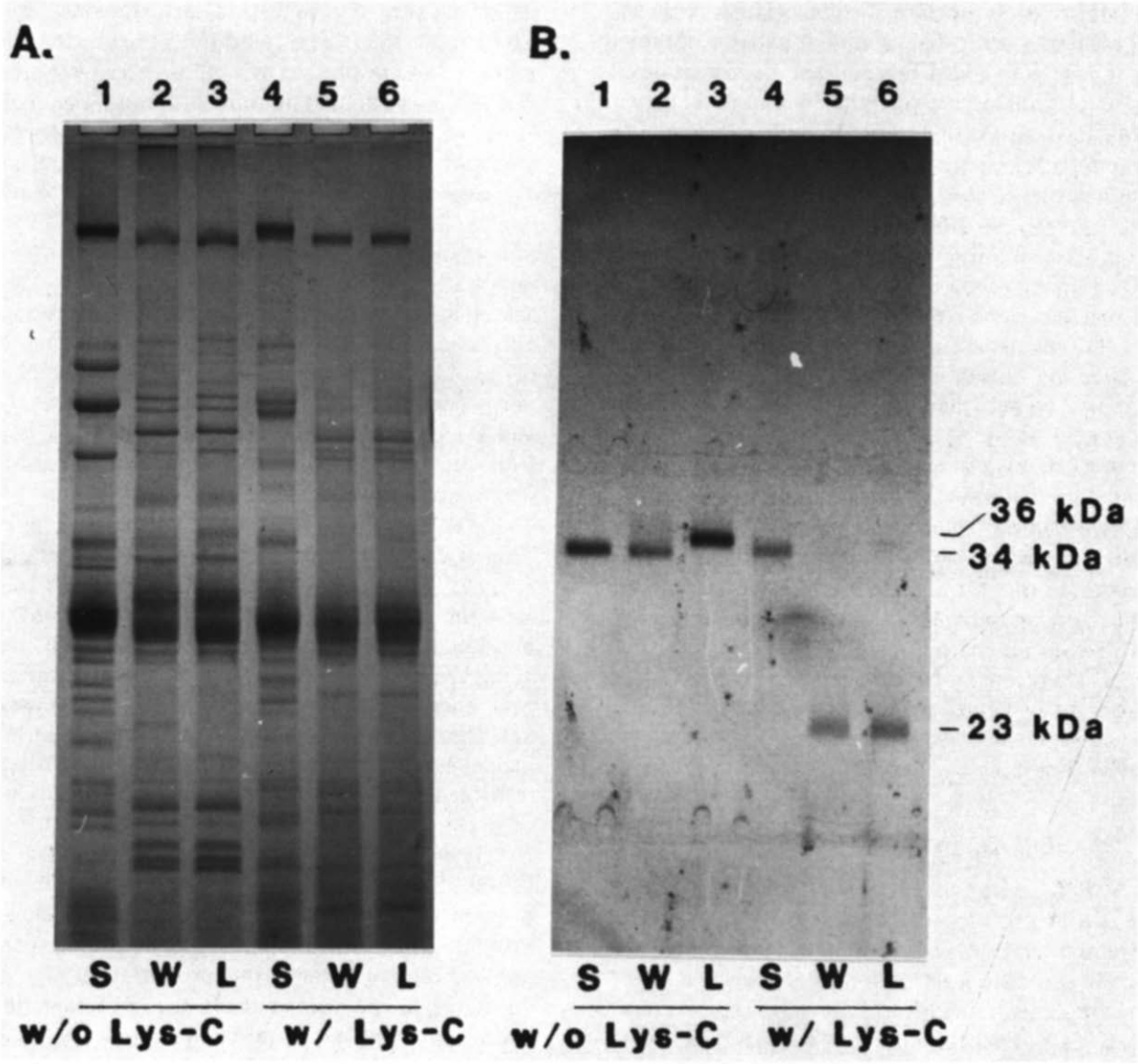

Fig.1. (A) Polypeptides revealed by LDS-PAGE of azido[^{14}C]atrazine-labeled thylakoid membranes of spinach (S), WT *Scenedesmus* (W), and the LF-1 mutant (L). Samples were incubated either without (w/o) or with (w) a lysine-specific protease (Lys-C) prior to electrophoresis. (B) Fluorograph obtained from the gel shown in A.

thylakoids were treated with a protease which cleaves polypeptide chains specifically at the carboxyl side of lysine residues. While the majority of the thylakoid proteins of the spinach sample (fig.1A, lane 4) are affected by the protease, there is no apparent change in the azido[^{14}C]atrazine-labeled 34-kDa band (fig.1B, lane 4). These data indicate that the spinach D1 protein has been labeled with azido[^{14}C]atrazine and also demonstrate the specificity of the protease (i.e. the lack of activity on a polypeptide lacking lysine). In contrast the labeled 34- and 36-kDa proteins of WT and LF-1 *Scenedesmus* are cleaved by the protease (lanes 5,6). In both cases the label is now associated with a band at ~23 kDa. This implies that the atrazine-binding protein in *Scenedesmus* contains one or more lysine residues. Only one labeled band was detected, indicating that the

radioactivity is specifically associated with the 23-kDa fragment. The identical pattern obtained with the WT and LF-1 mutant demonstrates a close relationship between the 34- and 36-kDa proteins and that the difference in molecular mass between them is associated with the non-labeled fragment.

Fig.2A shows polypeptide profiles of samples from spinach, WT *Scenedesmus*, and LF-1 mutant which are enriched in the PS II core complex. All of the samples have two bands in the 30–34-kDa region. The presence of the altered band at 36 kDa in the LF-1 mutant allows for identification of the proteins which had been radiolabeled in the thylakoid samples. These are labeled D1 in the figure (see also [13,23]). The other band in this region is probably the D2 protein [7]. A Western blot, obtained from a duplicate gel, was probed with antibodies against the D1 protein, and the results are shown in fig.2B. In each lane one major band is visible. The antibody recognized the spinach 34-kDa protein as well as the 34-kDa WT and the 36-kDa LF-1 mutant proteins. The other faint bands visible in fig.2B probably represent aggregation of D1 with itself or other proteins as well as possible degradation products. We noted previously [13] that one major and multiple minor radioactive bands could be detected in PS II core samples which had been isolated from azido[^{14}C]atrazine-labeled thylakoids even though only one radioactive band was apparent in the thylakoids. We have used PS II core samples in this experiment since it is difficult to detect the D1 and D2 proteins in thylakoid samples. It is clear from fig.2 that the D2 protein is not recognized by this antibody preparation.

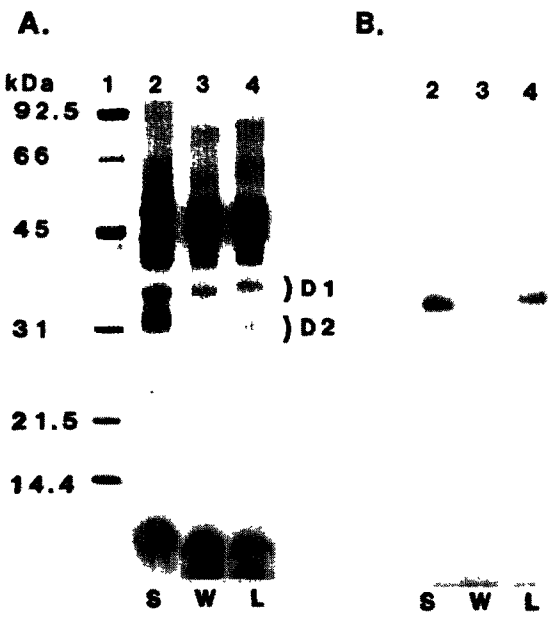

Fig.2. (A) Polypeptide profiles of PS II-core-enriched samples from spinach (S), WT *Scenedesmus* (W) and the LF-1 mutant (L). Protein molecular mass standards (lane 1) from Bio-Rad were: phosphorylase *b* (92.5 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21 kDa), lysozyme (14.4 kDa). (B) A Western blot (obtained from a duplicate of the gel shown in A) which has been probed with antibodies to the D1 protein of *A. hybridus*.

4. DISCUSSION

In the past, interest in the D1 protein has emphasized its involvement in binding different herbicides. The detection and sequencing of the *psbA* gene encoding this protein have allowed detailed analysis of the molecular basis for changes in herbicide binding and parallel effects on electron transfer to the plastoquinone pool in the absence of herbicides. This body of information can now be related to our understanding of the organization of the PS II core complex. In addition, our results provide a new perspective on the role of the D1 protein. The LF-1 mutant of *Scenedesmus* contains an altered form of this protein (figs 1,2), yet all of the phenotypic changes in the mutant have been associated with the oxidizing side of PS II, not the reducing side [10–13,24]. Until recently the possibility of functions for the D1 protein on both sides of the photosynthetic membrane would have seemed unlikely. However, the hydropathy plots of this protein suggest that its polypeptide chain spans the membrane several times [25]. Also, the D1 protein shows significant sequence homology to the L subunit of the purple bacteria reaction center [2–4]. The L protein is now known to have exposed segments on both sides of the membrane, and this is likely to be true for the D1 protein also. Our demonstration that the protein altered in the LF-1 mutant is the D1 protein provides evidence that it affects functions on the oxidizing side of PS II in addition to its known role on the reducing

side.

This conclusion allows us to rationalize more easily other data involving the D1 protein and the LF mutants. For example, it was noted that several independently isolated LF mutants show an identical change of the 34-kDa protein to one running at ~36 kDa, and it was hypothesized that the gene for a processing enzyme for this protein, rather than the gene coding for the protein itself, had been mutated [10]. This suggestion is now supported since a transient precursor of the D1 protein has been detected in vivo by pulse chase labeling and by in vitro protein synthesis [26,27]. The mobility difference between the precursor and mature form of the D1 protein is about 1.5 kDa [26,27]. Also, when the D1 protein is not synthesized (due to deletion of the *psbA* gene), a functional PS II core complex is not assembled [28]. The LF-1 mutant may represent a situation where the precursor form of the D1 protein is incorporated into the PS II complex but is not processed. We suggest that the presence of the D1 precursor in LF-1 allows the PS II complex to accumulate and to have both reaction center and reducing-side electron-transport activity.

It is known that the D1 protein turns over much more rapidly during illumination in vivo than other thylakoid proteins [29]. This has been linked to the specific loss of PS II activity during high intensity illumination. It has been suggested that production of reactive quinone anions on the reducing side of PS II results in the inactivation of the D1 protein, which is then selectively replaced in the complex [30]. However, several features of this PS II inactivation (e.g. loss of the variable component of the fluorescence yield and changes in the EPR signal II, see data in [30]) can also be interpreted as being associated with the loss of PS II oxidizing-side function. We suggest that these data can be rationalized more easily if one considers a dual function for the D1 protein.

It is possible that the phenotype observed in the LF-1 mutant is due to indirect effects of the non-processed portion of the D1 protein, e.g. steric hindrance may prevent binding of some Mn atoms to their functional site in the membrane. However, in the absence of evidence for indirect effects, we suggest that the D1 protein could play some direct role in the water-oxidation reactions and that the 'lumenal side' of the protein remains a prime candidate as the source of ligands to a portion of the Mn atoms involved in oxygen evolution.

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