The azido[^{14}C]atrazine photoaffinity technique labels a 34-kDa protein in *Scenedesmus* which functions on the oxidizing side of photosystem II

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We have used azido[^{14}C]atrazine to photoaffinity label thylakoids from wild-type (WT) *Scenedesmus* and a mutant, LF-1, which is blocked on the oxidizing side of photosystem II (PS II). One protein is labeled in each case, at 34 kDa in the WT and 36 kDa in LF-1. Previous comparison of the WT with LF-1 had been used to assign a PS II donor side function to the 34-kDa protein. These results suggest that this photoaffinity technique does not label the herbicide-binding protein involved in electron transfer on the reducing side of PS II.

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

1. INTRODUCTION

Herbicides such as DCMU and atrazine act by inhibiting electron transfer from a tightly bound quinone (Q_A) to a second quinone molecule (Q_B) on the reducing side of PS II [1,2]. It is believed that there is a binding site for these herbicides on an intrinsic membrane protein, called the Q_B-protein, which is a part of the PS II complex [3,4]. The herbicide–Q_B-protein interaction is non-covalent and does not survive strongly denaturing conditions such as those encountered during SDS-(or LDS-)PAGE. In an attempt to identify the band which contains the Q_B-protein after SDS-PAGE, Pfister et al. [5] developed a photoaffinity technique using a ^{14}C-labeled azido analog of atrazine (i.e., azido[^{14}C]atrazine). This technique has been used extensively, and it consistently labels a 32–34-kDa protein in thylakoids and PS II core complexes (e.g. [4–7]). The assumption that it labels the Q_B-protein has influenced the interpretation of much experimental data (e.g. [8–11]). However, there have been reports which suggest that the protein labeled by azido[^{14}C]atrazine may not be the Q_B-protein, or at least that more than one protein is involved in binding this type of herbicide [12,13].

A 34-kDa intrinsic protein, also a component of the PS II complex [14], has been identified in *Scenedesmus obliquus*, a green alga [15,16]. Analyses of low fluorescent (LF) mutants of this species have provided evidence that this protein functions on the oxidizing side of PS II [15–18]. For example, the mutant, LF-1, does not evolve O_2, is not blocked for electron transport on the reducing side of PS II, and contains a 36-kDa protein in place of the 34-kDa protein observed in the

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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; PQ, plastoquinone; PS, photosystem; Q_A, primary PS II quinone acceptor; Q_B, the secondary quinone acceptor of PS II; WT, wild type

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WT. Here we apply the azido[^{14}C]atrazine photoaffinity labeling technique to thylakoids from algal WT and LF-1 cells. Surprisingly, the technique labels the 34-kDa protein in the WT which has been implicated in PS II donor side function. This raises serious questions either about the efficacy of the technique for labeling the Q_B-protein or the true nature of the 34-kDa protein.

2. MATERIALS AND METHODS

WT *S. obliquus* and the LF-1 mutant were grown in the dark on liquid enriched medium [19]. Cells were broken and thylakoid membranes were collected and stored (−70°C) in 0.3 M sucrose, 50 mM NaCl, 5 mM $MgCl_2$, 50 mM Na/K phosphate buffer (pH 6.9) [19]. Labeling with azido-[^{14}C]atrazine (49.4 mCi/mmol, Pathfinders Lab, St. Louis) was performed according to [5]. Membranes were diluted to 50 μg Chl/ml in the storage solution, and azido[^{14}C]atrazine was added to yield a 4 μM solution of herbicide. The membranes were stirred at 5°C and irradiated for 30 min with a short-wavelength UV light (model UVSL-15, Ultra Violet Products, San Gabriel, CA) placed 5 cm above the liquid surface. Membranes were collected (10300 × *g* for 10 min) and homogenized in 10 mM Tricine, pH 8.0, 10% sucrose at 1 mg Chl/ml. A fraction enriched in PS II core particles was prepared from these labeled membranes as described in [14] except that, due to a limited amount of sample material, whole membranes, washed with 2 M NaBr, 40 mM Tricine, pH 8.0, were applied to the sucrose gradients rather than a detergent-derived PS II preparation. These fractions show some contamination with PS I and the cytochrome b_6/f complex.

LDS-PAGE [20] employed 10–20% acrylamide gradients or 10–17% gradients with 6 M urea included in the separating gel. Molecular mass standards were obtained from Bio-Rad. Gels were prepared for fluorography using EN^{3}HANCE (New England Nuclear), dried and exposed to X-OMAT AR film at −70°C for 3 or 4 weeks.

Fluorescence induction kinetics were monitored using a home-made, computer-accessed instrument similar to that described by Vernotte et al. [21]. Thylakoid membranes were diluted to 5 μg Chl/ml in 0.3 M sucrose, 10 mM NaCl, 25 mM Hepes, pH 7.4. Final concentration of additions to the thylakoids were 3.0 mM for NH_2OH and 10 μM for atrazine (CIBA-Geigy).

3. RESULTS

Fig.1A and B shows fluorescence induction traces for thylakoids isolated from WT *Scenedesmus* and from the LF-1 mutant, respectively. Fluorescence of WT membranes shows the expected rapid rise to an F_o value followed by a slower rise to F_{max} as the PQ pool becomes reduced. Addition of 10 μM atrazine results in a much more rapid rise to the F_{max} value indicating electron transfer is blocked between Q_A and the PQ pool (e.g. [2]). LF-1 thylakoids show a similar F_o value, but have lost the variable fluorescence component. Addition of a PS II electron donor, in this case

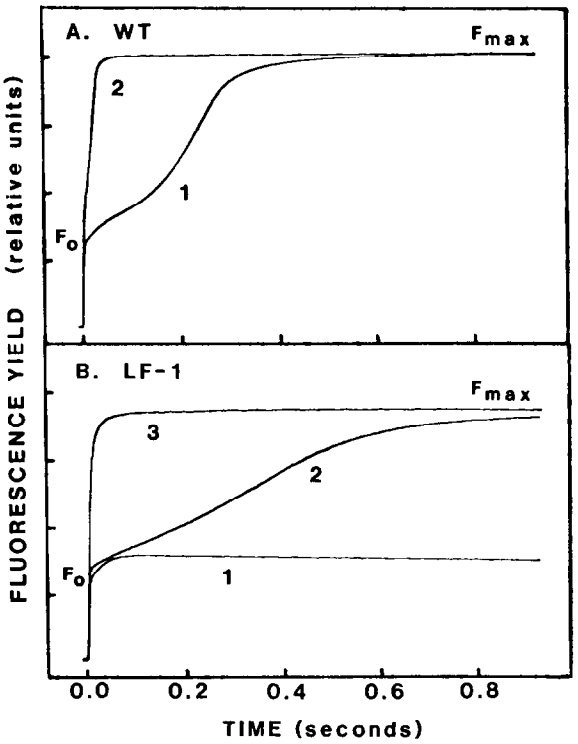

Fig.1. Fluorescence induction curves for thylakoid membranes from WT *Scenedesmus* (A) and the LF-1 mutant (B). Membranes, at 5 μg Chl/ml, were dark adapted for 10 min prior to illumination. (A) Trace 1, no additions to the membranes; 2, membranes from 1 plus 10 μM atrazine. (B) Trace 1, no additions to the membranes; 2, fresh membranes plus 3.0 mM NH_2OH; 3, samples from 2 plus 10 μM atrazine.

NH_2OH, leads to the restoration of a variable fluorescence. The rate of electron donation from NH_2OH to PS II is slower than that of the native water-splitting system, and this is reflected by the slower rise of the fluorescence yield. These data are indicative of a lesion on the water-splitting side of PS II before the point of electron donation to PS II [22,23]. Addition of 10 μM atrazine to the LF-1 thylakoids (with 3.0 mM NH_2OH) results in a rapid rise to the F_{max} level, i.e., the atrazine is blocking electron transfer on the reducing side of PS II.

Fig.2A shows the polypeptides of azido[^{14}C]-atrazine-labeled thylakoids from WT *Scenedesmus* and LF-1 as revealed by Coomassie brilliant blue staining of an LDS-polyacrylamide gel. The shift in mobility of a protein, from 34 kDa in the WT to approx. 36 kDa in LF-1, can be seen in these samples. Additionally, the LF-1 thylakoids shown here have lost a 17-kDa protein and are reduced in a 23-kDa protein (indicated with arrowheads). These are extrinsic proteins which have been implicated in the water-splitting reactions [24,25]. Previous electrophoretic analysis had not indicated a loss of proteins with these molecular masses in thylakoids of LF-1 [15,17]. Data to be presented elsewhere suggest that treatments which expose the lumenal side of thylakoids to the buffering medium may result in the loss of the 17- and 23-kDa proteins from LF-1 membranes (e.g., Triton X-100-derived inside-out PS II preparations or prolonged stirring of thylakoid samples such as that used in azido[^{14}C]atrazine labeling).

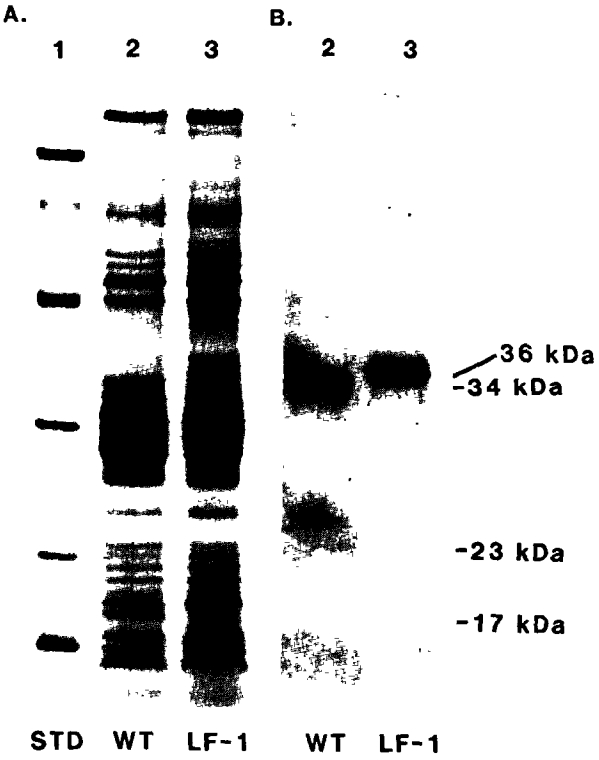

Fig.2. (A) Polypeptides revealed by LDS-PAGE of azido[^{14}C]atrazine labeled thylakoid membranes from WT *Scenedesmus* and LF-1 mutant. Lanes: 1, molecular mass standards; 2, WT thylakoids; 3, LF-1 thylakoids. (B) The fluorograph obtained from the gel shown in A. Protein standards were: phosphorylase *b*, 94 kDa; bovine serum albumin, 68 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; soybean trypsin inhibitor, 21 kDa; lysozyme, 14.3 kDa.

Fig.2B is the fluorograph obtained from the gel of fig.2A. One major band was labeled with azido[^{14}C]atrazine in each case, a 34-kDa protein in WT and a 36-kDa protein in LF-1 thylakoids.

Fig.3 shows the polypeptides of samples enriched in PS II core complexes from WT *Scenedesmus* and LF-1. These samples were derived from the azido[^{14}C]atrazine labeled thylakoids used in fig.2. Several of the major polypeptides have been identified, including: the apoproteins of the two Chl *a*-protein complexes of PS II (PS II/Chl *a*), and the major protein of cytochrome *b*-559. The PS I reaction center Chl-protein complex (CPI) and its apoprotein (CPI'), which are present as contaminants in these preparations, are also indicated.

As we reported previously [14], the 34- and 36-kDa proteins are present in PS II core complexes from the WT and LF-1, respectively (fig. 3A). The fluorograph of this gel demonstrates that these same proteins are labeled by azido[^{14}C]atrazine. The faint bands present in the fluorograph probably arise from the 34- and 36-kDa proteins, either as multimers or as complexes with other proteins. They were not observed in the fluorograph of the original thylakoid preparation (fig.2B), from which these samples were derived. The tendency of this hydrophobic protein to form aggregates has been noted previously [14,15].

In both WT and LF-1 PS II core samples, there is a diffusely stained area visible with an apparent molecular mass of 30–32 kDa (fig.3A). Satoh et al. [7] using PS II core particles from spinach

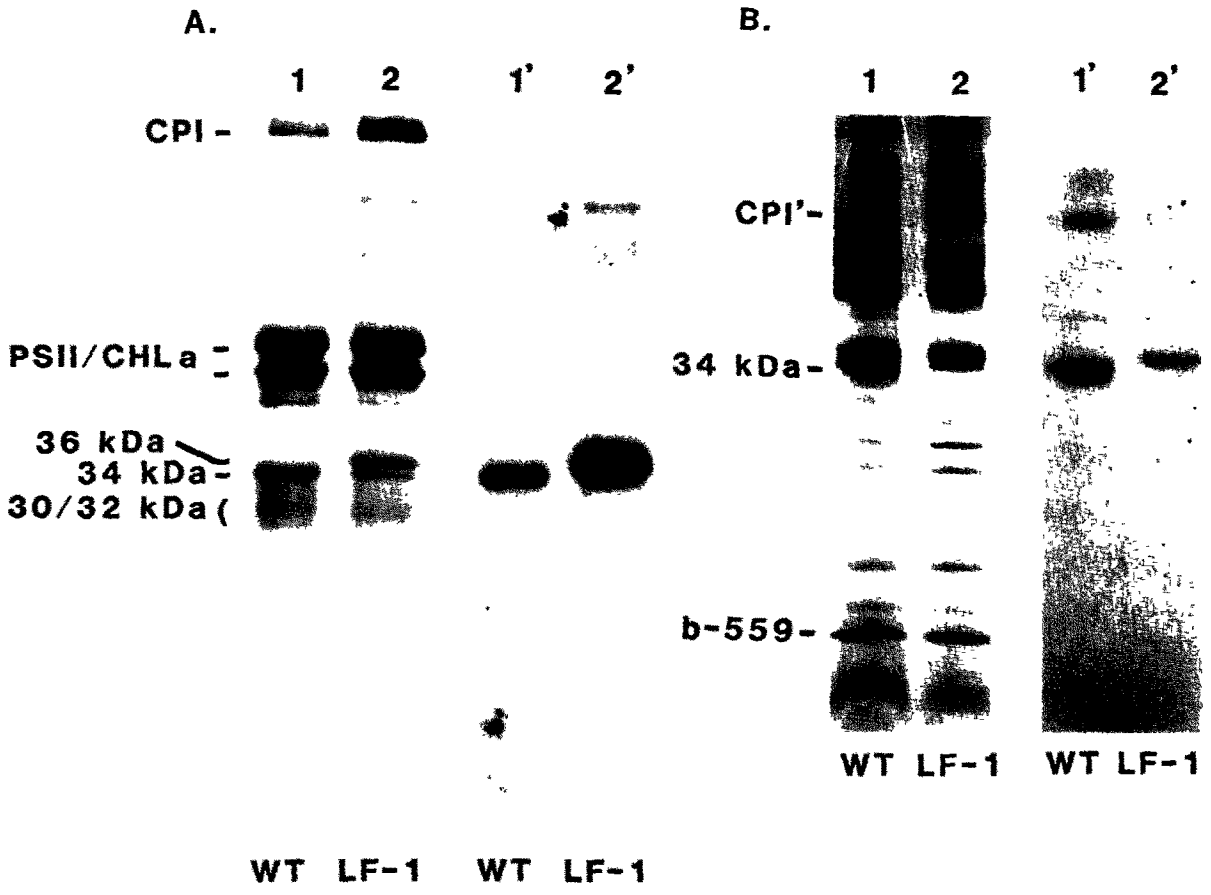

Fig.3a. Coomassie brilliant blue-stained polypeptides and fluorographs (') of PS II core enriched samples derived from azido[^{14}C]atrazine labeled thylakoids of WT *Scenedesmus* and the LF-1 mutant. (A) Separation was by LDS-PAGE without urea. (B) Separation was by LDS-PAGE with 6 M urea in the gel.

found that inclusion of urea (>4 M) in SDS gels allows for the resolution of two distinct bands in this region and that it is the faster moving band which is labeled by azido[^{14}C]atrazine. Fig.3B shows the pattern obtained when the PS II core samples from WT and LF-1 are separated on gels containing 6 M urea. Two distinct 30–34-kDa bands are now observed in the WT sample. Comparison of the WT and LF-1 samples identifies the lower band as the one which has an apparent molecular mass of 34 kDa in gels without urea. This is further indicated by the fluorograph (fig.3B). As reported for the spinach preparation [7], it is the lower band which is labeled by azido[^{14}C]atrazine. Again, a difference in mobility of the azido[^{14}C]atrazine labeled proteins of WT and LF-1 is observed. The upper band seen in the WT sample may be the same protein which runs as the indistinct 30–32-kDa zone in gels without urea, although this has not been demonstrated definitively at this time. Only one stained band is observed in the LF-1 sample (fig.3B, lane 2) due to comigration of the two proteins observed in the WT sample. The presence of urea also increases the resolution in the lower portion of the gel. The major polypeptide of cytochrome *b*-559 which was not visible in fig.3A can now be identified at ~10 kDa.

4. DISCUSSION

The fluorescence induction data presented here and in [16] indicate that the LF-1 mutant has a functional Q_B-protein. Therefore we expected the azido[^{14}C]atrazine photoaffinity technique to label identical proteins in the WT *Scenedesmus* and

LF-1. However, as shown in fig.2, it labels a 34-kDa protein in the WT and one at 36 kDa in LF-1. The difference in mobility of these two labeled proteins allows identification of the 34-kDa WT protein as one suggested to have a function on the donor side of PS II.

The mobility characteristics of the labeled protein from WT *Scenedesmus* on gels with and without urea (fig.3) are similar to those reported in spinach PS II core particles [7]. This implies that equivalent proteins are labeled in higher plants and the green algae. We suggest that this technique, in general, labels proteins homologous to the 34-kDa protein identified in *Scenedesmus*. Assignment of a donor side role to the 34-kDa protein has relied, in part, on analysis of the LF-1 mutant. The loss of water-splitting activity in LF-1 has been correlated with the alteration of the normal 34-kDa protein in the WT to 36 kDa in the mutant. We have noted that thylakoids of LF-1 shown in fig.2A are depleted in two extrinsic proteins which have a role in the water-splitting reactions. However, based on the current understanding of the function of these proteins [26], their loss alone cannot account for LF-1's phenotype (i.e., total lack of oxygen-evolving capability). Also, other electrophoretic studies have not revealed differences between WT *Scenedesmus* and LF-1 in these regions of the gels [15,17]. We believe these proteins are present in intact thylakoids of LF-1 and have been lost in this case during the labeling procedure.

The chloroplast gene which codes for the Q_B-protein has been identified and cloned from maize [27,28]. It is designated the *psb*A gene and has been used as a probe to identify homologous genes in other species. The demonstration that mutants which show resistance to atrazine have a consistent alteration in the nucleotide sequence of the *psb*A gene provided evidence linking this gene to the Q_B-protein [4,6,29]. The nucleotide sequence of the *psb*A gene has been used to predict a molecular mass for the Q_B-protein of approx. 34 kDa [29]. However, it is not clear from the literature that the protein product of the *psb*A gene is labeled by the azido[^{14}C]atrazine photoaffinity technique. PS II core complexes isolated from a variety of sources have been found to contain at least two proteins with molecular masses in the 32–34-kDa range [7,14,30,31]. One of these is likely to be the Q_B-protein and one may function on the donor side. Two-dimensional models of PS II typically show proteins involved in water oxidation restricted to the lumenal side of the membrane while the Q_B-protein is located towards the stromal side. However, recent analyses of intrinsic membrane proteins have indicated that their polypeptide chains will span the membrane several times [4,32]. It is quite possible and even likely that portions of Q_B-protein and the 34-kDa protein will be located very close to each other at some point in the membrane. Furthermore, structural influences on activity may dictate conservation of a particular orientation. The nitrene group formed by UV irradiation of azidoatrazine will bind to whatever protein it is nearest. This does not necessarily have to be the same protein which contains the actual binding site for azidoatrazine. Also, it is possible that more than one protein influences the binding of triazine herbicides. Others have mentioned these possibilities. For example, Oettmeier et al. [12] suggested that a photoaffinity analog of plastoquinone preferentially labels a membrane protein in the 30–34-kDa range which is different from that tagged by azido[^{14}C]atrazine. Renger et al. [13] presented data which implicate a protein with exposed lysine residues as affecting herbicide binding (nucleotide sequence data indicate the Q_B-protein has no lysine residues [4,6,29]). Also it was shown that azidoatrazine labels the L subunit of *Rhodopseudomonas sphaeroides* reaction centers [9] even though immunological data had indicated that the M and H subunits are involved in Q_B binding [33].

From data presented here, we suggest that the azido[^{14}C]atrazine photoaffinity technique does not label the Q_B-protein but in fact labels the 34-kDa protein, which functions on the oxidizing side of PS II. Although other interpretations may be possible, this result suggests that conclusions based on the use of this technique in previous studies may have to be reassessed.

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