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Characterisation of the D1 protein in a photosystem II mutant (LF-1) of *Scenedesmus obliquus* blocked on the oxidising side

Evidence supporting non-processing of D1 as the cause of the lesion

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The D1 polypeptide of the photosystem II reaction centre in a mutant (LF-1) of *Scenedesmus obliquus* lacking a water-splitting manganese complex is approx. 1.5 kDa larger than that in the wild type but the D2 protein is the same size. The peptide profiles of D1 on partial digestion with papain or endoproteinase Lys-C indicate that the extra segment in the LF-1 protein is located at or near the carboxyl-terminus. The D1 proteins produced by in vitro translation of mRNA from wild type and LF-1 cells have an identical molecular mass to D1 from LF-1 thylakoids whereas D1 from wild-type thylakoids is 1.5 kDa smaller due to C-terminal processing. These results support the hypothesis that in the LF-1 mutant, the D1 protein is incorporated into the PS II reaction centre, but the C-terminal extension is not removed.

Carboxyl-terminal processing; D1 polypeptide; Photosystem II; *psb*A gene product; (*Scenedesmus obliquus*)

1. INTRODUCTION

It is now widely accepted that the primary photoreactants of PS II[3] are bound to a heterodimer of the D1 and D2 proteins analogous to the L and M subunits of the bacterial reaction centre [1–5]. Recent data suggest that one or both of these polypeptides provides ligands for the manganese cluster involved in water oxidation, whereas the extrinsic 33, 23 and 17 kDa polypeptides attached to the lumenal surface of the PS II core complex stabilise the cluster and maintain an optimal conformation for maximal rates of water oxidation [6–9]. However, very little is known about how the PS II complex is assembled, or precisely where the manganese is bound.

Metz et al. [10] have characterised a low-fluorescence mutant (LF-1) of *Scenedesmus obliquus* which provided some of the first evidence that D1 may be involved in manganese binding. Although it lacks the ability to evolve oxygen [10], PS II activity is restored when artificial donors are added [11], and the presence of the primary acceptor quinone $Q_A^-Fe^{2+}$, and the secondary donor signal II_s (D^+) have been detected by EPR [12]. In addition, the mutant contains less than half the normal manganese content per PS II complex, signal II_s saturates at a lower microwave power, and the cytochrome *b*-559 is in its low-potential form [10,12], all symptomatic of the absence of the

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Abbreviations: PS II, photosystem II; LF-, low fluorescence; C-terminus, carboxyl-terminus of protein; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; azidoatrazine, 2-azido-4-ethylamino-6-isopropylamino-*s*-triazine

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tetranuclear manganese cluster involved in water oxidation. Immunoblotting experiments have shown that in LF-1, the D1 polypeptide has an increased apparent molecular mass of 36 kDa compared to 34 kDa in the wild-type [13]. In other algae and higher plants, it has been shown by pulse-labelling experiments and by in vitro protein synthesis that the D1 protein is synthesised as a precursor which has an apparent molecular mass 1.5 kDa greater than that of the processed form [14–16]. Processing is thought to be by cleavage near the C-terminus [17]. It has been suggested that in LF-1, D1 is incorporated into the PS II complex but is not processed and that this results in loss of donor side activity [13]. Here, we have confirmed the immunoblotting results on D1 in LF-1 and we have been unable to detect any changes in D2 from LF-1 compared to the wild type. Additionally, we present evidence consistent with the hypothesis that D1 from the LF-1 mutant possesses a C-terminal extension which is normally removed.

2. MATERIALS AND METHODS

The wild type and LF-1 mutant of *S. obliquus* strain D_3 were grown in dim light (approx. 1 $W \cdot m^{-2}$) in an enriched medium, as described [18]. Both strains were provided by Dr N. Bishop (Oregon State University). Thylakoids were prepared from algal cultures as in [19]. Spinach core complexes were prepared as described [20].

The D1 polypeptide was labelled in vivo with [^{35}S]methionine (Amersham) by an adaptation of the procedure described in [16]. 100 ml mid-log phase cultures were harvested and resuspended in 100 ml sulphate-free growth medium. After 24 h adaptation the cultures were gently sedimented and resuspended in 10 ml of the supernatant. After 30 min adaptation in dim white light (approx. 1 $W \cdot m^{-2}$), 100 μCi [^{35}S]methionine (>800 Ci/mol) was added and the culture was incubated for 4 h at 24°C with shaking under 5 $W \cdot m^{-2}$ illumination before thylakoid preparation.

SDS-PAGE of thylakoids or in vitro translation products was performed as in [21] on 7–17% (w/v) gradient gels. Samples were solubilised at room temperature in sample buffer prior to loading. Partial proteolytic digestion experiments with papain were performed as in [2,22]. For digestion with endoproteinase Lys-C, the region of the gel containing the [^{35}S]methionine-labelled D1 was excised and the protein electroeluted essentially as described [23]. Following electroelution the protein solution

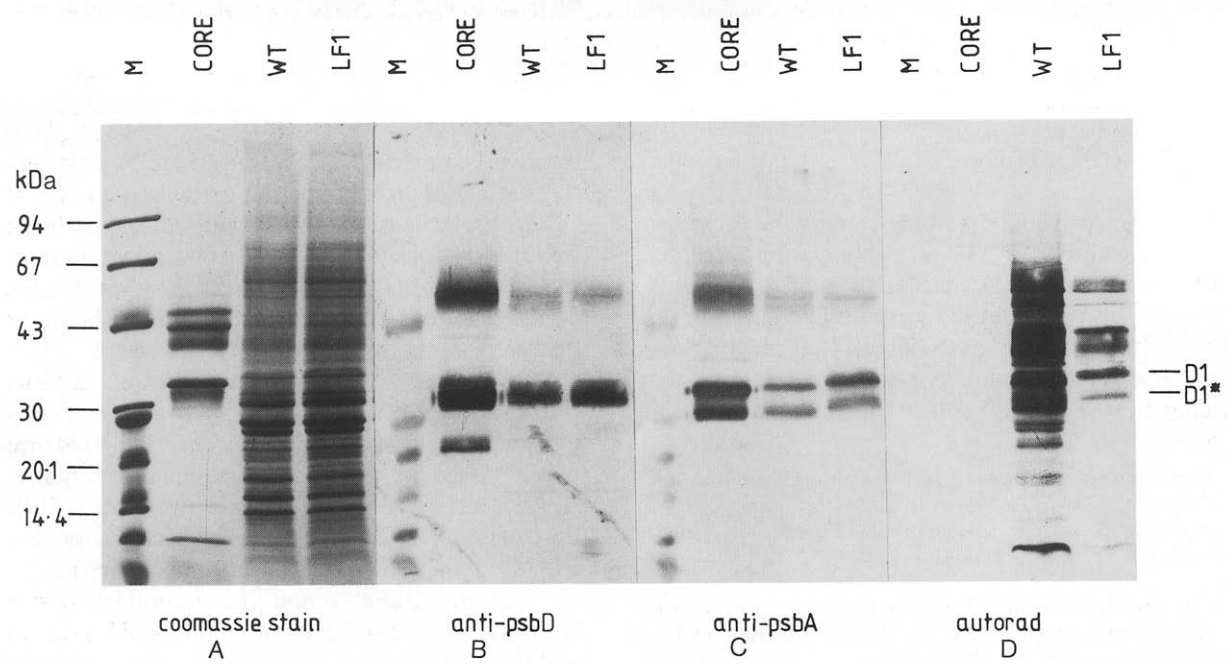

Fig.1. Immunological analysis of thylakoid membrane proteins from wild-type (WT) and the low fluorescence (LF-1) mutant of *Scenedesmus obliquus*, using antibodies specific for the *psb*D and *psb*A gene products (B,C). For comparison, PS II core particles from spinach were run in parallel. (A) Coomassie blue stained pattern of polypeptides in the thylakoid membrane following SDS-PAGE; (D) fluorograph of a similar gel after in vivo labelling of *Scenedesmus* with [^{35}S]methionine. The positions of D1 and D1*, the putative D1 conformers, are indicated.

was concentrated using Amicon Centricon 10 microconcentrators and digested with the endoproteinase Lys-C (Boehringer Mannheim) as described in [24]. Gels were fixed, stained, destained and prepared for fluorography using Amplify (Amersham). For immunoblotting experiments, proteins separated by SDS-PAGE were transferred onto nitrocellulose (pore size 0.2 μm). Blots were then probed with purified antibodies to the *lacZ/psbA* gene fusion product (D1–β-galactosidase fusion protein) and the *lacZ/psbD* gene fusion product (D2-β-galactosidase fusion protein) prepared as in [25]. Bound antibody was visualised using alkaline phosphatase conjugated to goat anti-rabbit antibodies (Promega).

The [^{35}S]methionine-labelled precursor of the D1 polypeptide was obtained by in vitro translation of mRNA. Total cellular RNA was extracted according to Koller et al. [26] from 400 ml mid-log cultures of LF-1 and wild-type cells grown as above. The mRNA encoded by the *psbA* gene was affinity purified by hybrid selection [27] using 250 μg total cellular RNA and 10 μg linearised plasma DNA immobilised on Gene Screen (New England Nuclear). The plasmid was constructed by cloning a 2.2 kb *Eco*RI fragment containing the *psbA* gene from *Poa annua* into pUC9 [28]. The hybridised RNA was eluted and translated in vitro using a rabbit reticulocyte lysate system (Amersham). The D1 polypeptide was then immunoprecipitated using the antibodies specific for the *psbA* gene product, and analysed by SDS-PAGE and fluorography as above.

For Northern blot analysis, 5 μg total cellular RNA isolated as above was subjected to electrophoresis on a 1.8% agarose gel containing formaldehyde as described in [29]. RNA was transferred onto a nitrocellulose sheet, hybridised to a ^{32}P-labelled DNA probe for D1 and washed as in [30]. The probe was a 1.2 kb *Bam*HI fragment carrying the *psbA* gene from *P. annua* isolated from the plasmid pAS3-91 (a kind gift from Dr M.D.C. Barros). It was labelled to high specific activity as in [31].

3. RESULTS

As previously observed [13], in the LF-1 mutant, D1 has an apparent molecular mass 1–2 kDa greater than in the wild type (fig.1). The anti-D1 antibody showed a strong cross-reactivity with a polypeptide of apparent molecular mass 32–34 kDa in the wild type, assumed to be the D1 polypeptide (panel C). Other reactive polypeptides with apparent molecular masses of 29 and 55 kDa were also observed. An immunoreactive species of 55 kDa has also been observed in extracts of *Spirodela oligorrhiza* [32], and in isolated PS II reaction centre preparations from pea [2] and has been assumed to be an aggregate containing D1. A cross-reacting species with an apparent molecular mass of 29 kDa has also been observed previously

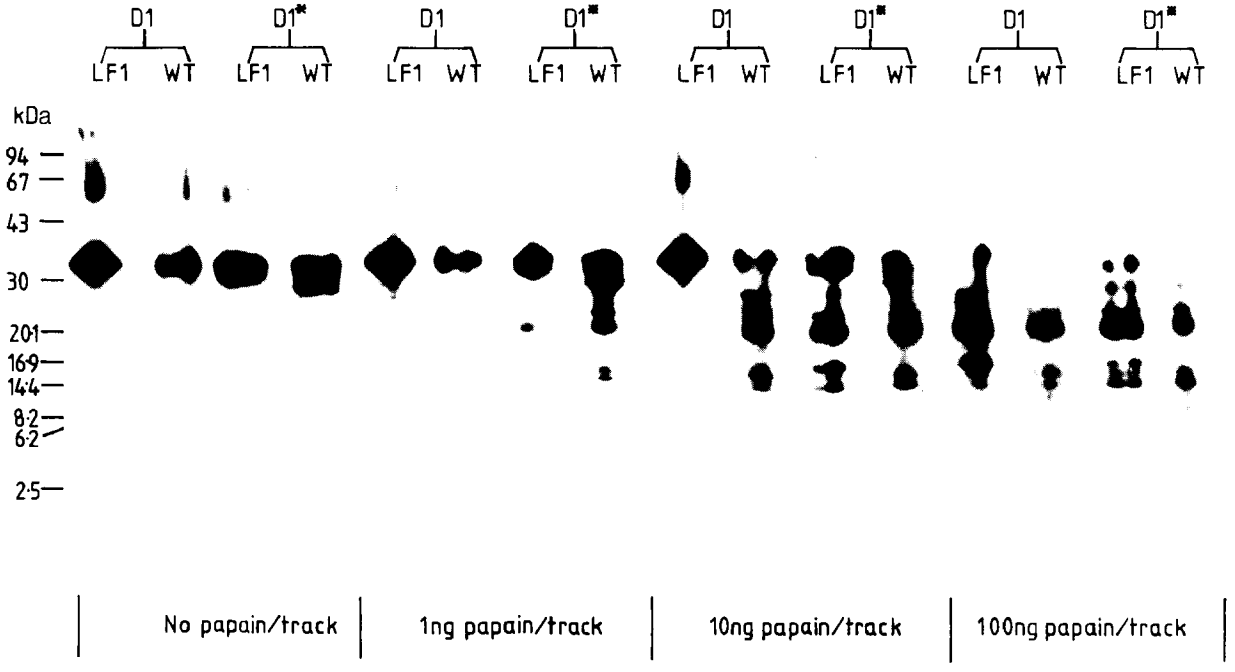

Fig.2. Partial proteolytic analysis of the putative D1 conformers from wild-type (WT) and LF-1 strains of *S. obliquus* by the method of Cleveland et al. [22]. The ^{35}S-labelled polypeptides D1 and D1* (see fig.1) were separated by SDS-PAGE and re-electrophoresed in the presence (1–100 ng per track) or absence of papain.

[2,32]; it was suggested to be a conformer of D1. This possibility was examined in more detail (see below). The antibody raised against the D2 polypeptide cross-reacted with a protein band of 30 kDa in both the wild-type and LF-1 mutant (fig.1B). Cross-reactivity was also observed with a protein band of this apparent molecular mass in PS II core particles from spinach. Cross-reactivity with a higher molecular mass species (again 55 kDa) probably indicated that this species was a D1/D2 heterodimer. A fluorograph of the [^{35}S]methionine-labelled thylakoids (panel D) revealed that the polypeptides which cross-reacted with the anti-D1 antibody were also heavily labelled with ^{35}S which is consistent with the well-known rapid incorporation of label into D1 under the conditions employed here [16]. When the gel slices containing the two putative conformers of D1 were excised and re-electrophoresed, they had the same apparent mobility (fig.2).

On treatment of *Scenedesmus* D1 with limiting amounts of papain, a rather similar pattern of proteolytic digestion products was observed in this study (fig.2) to that previously reported for D1 from *Spirodela* [17]. Treatment of the two putative D1 conformers gave rise to the same partial proteolytic digestion pattern, implying that they were indeed identical. It is significant, however, that there were some differences in the pattern derived from the wild-type protein and that from the LF-1 mutant. In particular, the D1-derived peptide that has an apparent molecular mass of 14 kDa in the wild type had an increased molecular mass of 16 kDa in the LF-1 mutant.

Endoproteinase Lys-C cleaves polypeptides specifically at the carboxyl side of lysine residues. D1 purified by electrophoresis from wild-type and LF-1 thylakoids was subjected to digestion with Lys-C. For both strains, cleavage with Lys-C resulted in the formation of two fragments (fig.3), one with an apparent molecular mass of 23 kDa. The other fragment had a molecular mass of 13 kDa when derived from wild-type D1 and 15 kDa when derived from LF-1 D1.

If the LF-1 mutant arises as a consequence of failure to process D1, two implications are that (i) the D1 precursor should be the same in the wild type and LF-1 mutant and (ii) the mRNA that codes for the D1 precursor should be the same in both strains. The precursor can be generated by in

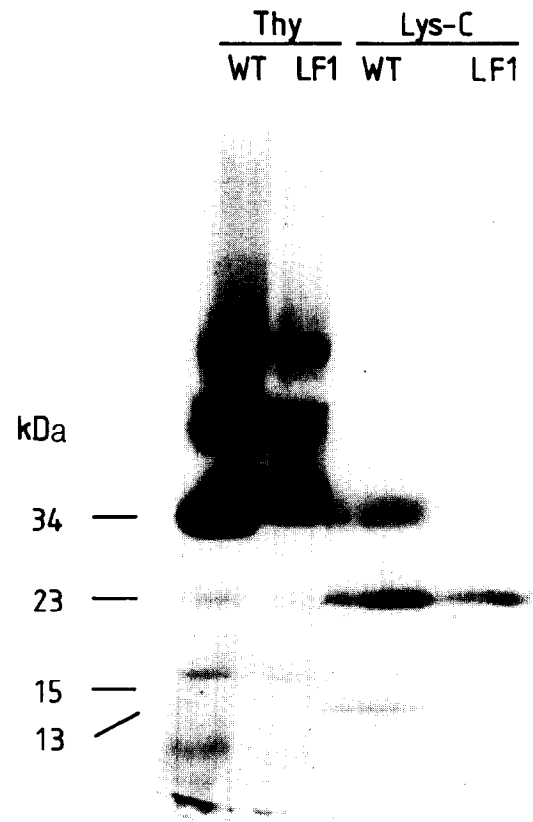

Fig.3. Proteolytic analysis of D1 from WT and LF-1 strains of *S. obliquus* using the lysine-specific endoproteinase Lys-C. [^{35}S]Methionine-labelled D1 was purified by SDS-PAGE and treated with Lys-C at 100 μg/ml. The proteolytic profile was visualised by SDS-PAGE and fluorography (tracks 3,4). Tracks 1,2 show a fluorograph of [^{35}S]methionine-labelled thylakoid proteins after in vivo labelling.

vitro translation of the mRNA species that codes for D1.

Analysis of the translation products of hybrid selected D1 mRNA by immunoprecipitation with the anti-D1 antibody followed by SDS-PAGE and fluorography can be seen in fig.4. In both the wild-type and LF-1 strains a protein with an apparent molecular mass of 34–36 kDa was immunoprecipitated (tracks 2,3). When mRNA was omitted from the rabbit reticulocyte lysate system this band was not detected by immunoprecipitation (track 1). Other lower molecular mass polypeptides were also immunoprecipitated but these species were also present in the minus RNA control track. This result strongly implies that the

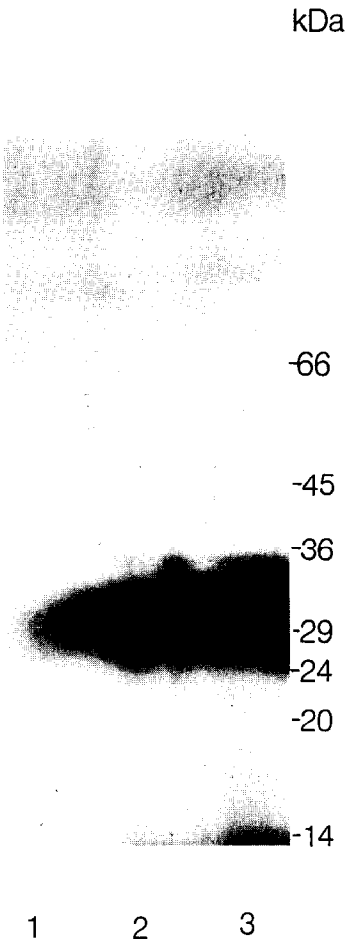

Fig.4. Immunoprecipitation of the in vitro translation products of hybrid selected mRNA. The mRNA species that codes for the *psb*A gene product was selected by hybridisation with linearised plasmid and translated using a rabbit reticulocyte lysate system (Amersham). The translation products were immunoprecipitated with antibodies specific for the *psb*A gene product and visualised by fluorography. Tracks: 1, no RNA control; 2, LF-1; 3, WT.

wild-type D1 precursor is of the same molecular mass as LF-1 D1 in the thylakoid membrane and that LF-1 D1 is not processed.

In the Northern blot analysis (fig.5) only one RNA species hybridised to the *psb*A gene probe. Within the limits of accuracy of this method the cross-reacting RNA species was of the same size in LF-1 and wild type (approx. 1.3 kb). This is of a similar size to the *psb*A transcript in *Sp. oligorrhiza* [33], *Euglena gracilis* [34] and *Chlamydomonas* [35].

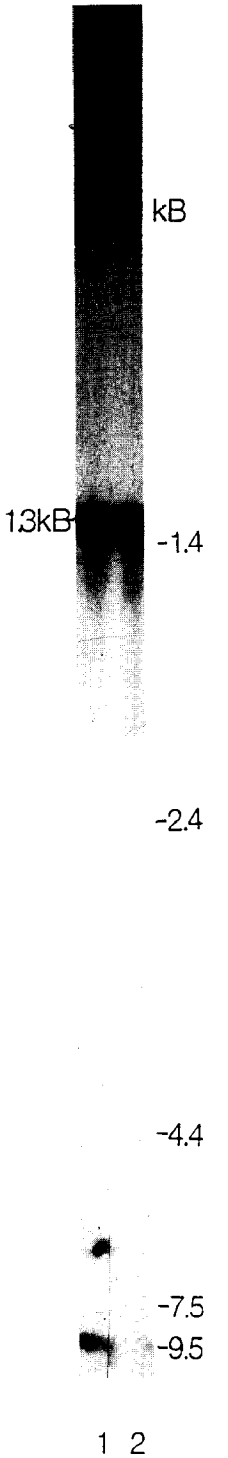

Fig.5. Northern analysis of mRNA from wild-type and LF-1 strains of *S. obliquus*. 5 μg total RNA from each strain was separated on a 1.8% agarose gel in the presence of formaldehyde and hybridised to a ^{32}P-labelled DNA probe for the *psb*A gene. The position of RNA markers is indicated. Tracks: 1, WT; 2, LF-1.

4. DISCUSSION

Characterisation of the LF-1 mutant of *S. obliquus* has previously revealed that a change in molecular mass of the D1 polypeptide of PS II is accompanied by a loss of donor side function [13]. Here, we have confirmed this observation and extended it to show that D2 from the LF-1 mutant did not have a different mobility from that of the wild-type protein. Since there is no evidence to suggest that D2 is processed from a larger precursor, this observation is consistent with the hypothesis that the failure to cleave uniquely D1 is responsible for the mutant phenotype in LF-1.

We have also shown that both processed D1 and the larger D1 protein from LF-1 exist in two conformers. As well as being present in *Scenedesmus*, such conformers have been observed previously in pea [2] and *Spirodela* [32] and hence appear to be a general phenomenon. This emphasises the importance of immunoblotting experiments in correctly assigning bands on SDS-PAGE gels to the various PS II polypeptides.

From the similarity in molecular mass of the precursor form of D1 from spinach and of D1 from thylakoids of the LF-1 mutant, it has been suggested that in the mutant the C-terminal processing of D1 is defective and the precursor form of the protein is incorporated into PS II [13]. We have tested this hypothesis by analysis of the peptides produced on partial proteolytic digestion of D1. Previously, Marder et al. [17] have shown that one of the cleavage products of D1 produced on treatment with low concentrations of papain is located at the C-terminal end of the D1 protein. From the similarity of the digestion pattern of D1 from *S. obliquus* with that obtained previously in *Spirodela*, we can conclude that this fragment is equivalent to the 14 kDa peptide from wild-type thylakoids and the 16 kDa peptide from LF-1 thylakoids. This strongly suggests that, in the LF-1 mutant, D1 has a C-terminal extension.

This result was supported by the experiments using the endoproteinase Lys-C. Previously, it has been shown that azidoatrazine covalently labels a 23 kDa fragment formed on Lys-C treatment of D1 and that this fragment has the same apparent molecular mass in LF-1 and wild type [13]. The remarkably highly conserved D1 protein from many plants is unusual in being totally devoid of lysine residues, but in D1 from both *Glycine max* [36] and *P. annua* [37], Arg-238 is replaced by lysine. In spinach D1, azidoatrazine labels Met-214 and a residue between His-215 and Arg-225 [38]. The size of the azidoatrazine-tagged Lys-C fragment from *Scenedesmus* D1 suggests that the Lys-C site could be at residue 238. This would place the lower molecular mass fragment at the C-terminus. The increased molecular mass (by 1.5 kDa) of this fragment in the LF-1 thylakoid D1 in comparison to that from the wild-type mature protein is therefore consistent with a failure to remove a C-terminal extension in LF-1.

The above conclusions support the hypothesis that D1 in PS II from the LF-1 mutant is present in the precursor form. Furthermore, the fact that the D1 precursor obtained by in vitro translation of RNA isolated from both wild type and LF-1 was of the same molecular mass as D1 in LF-1 thylakoids is consistent with the non-processing hypothesis. Finally, the mRNA that encodes for the D1 precursor was shown to have the same electrophoretic mobility in both wild-type and LF-1 cells. Our results support the proposal that a failure to remove an extension from the C-terminus of D1 does not prevent its integration into the PS II complex, but in some way prevents the formation of a complete manganese water-splitting complex.

The C-terminus of D1 is thought to be located at the lumenal side of the thylakoid membrane [3,39]. This extension may therefore sterically hinder the manganese-binding domain. However, we cannot at this stage preclude the possibility that a failure to remove the extension affects assembly of the manganese complex in a less direct way perhaps by affecting the binding of the extrinsic polypeptides. Blotting experiments with antibodies to the spinach 33 kDa extrinsic protein involved in stabilising the manganese complex [6,7] indicate that this protein is present in both LF-1 and wild-type thylakoids [40], although we cannot be sure that it is properly attached to the reaction centre in the former case. Proteins equivalent to the 23 kDa and 17 kDa extrinsic proteins which maintain the oxygen-evolving complex in an optimum conformation for maximal rates of water oxidation [9], possibly by creating high-affinity binding sites for Ca^{2+} and Cl^- [8] have been reported to be present in LF-1 thylakoids, although at variously diminish-

ed levels in comparison to the wild type [11]. Tamura and Cheniae [41] have shown that after treatment of PS II membranes to remove the tetranuclear manganese complex and the extrinsic proteins, water-oxidation activity can be restored by incubating the membranes in the light in the presence of $MnCl_2$ and $CaCl_2$ but in the absence of the extrinsic proteins. We have so far been unable to restore any water-oxidation activity to LF-1 PS II membranes using this protocol, which does however, photoactivate water oxidation in PS II membranes from dark-grown wild-type cells (Packer, J. et al., unpublished). This suggests that the failure to remove the C-terminal extension may itself be responsible for blocking assembly of the manganese cluster, at least during photoactivation in vitro.

A failure to process D1 in the LF-1 mutant could result from an absence of the processing enzyme or an alteration in the amino acid sequence of the D1 protein which blocks processing. We are at present carrying out further experiments including sequencing the C-terminal regions of the thylakoid-derived D1 proteins from both strains in order to shed further light on this question.

During the preparation of this manuscript, it came to our attention that Diner et al. [42] have performed a study similar to ours on the LF-1 mutant, and have come to the same conclusion concerning the nature of the altered D1 protein in this mutant.

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