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Processing of the D1 polypeptide of the photosystem II reaction centre and photoactivation of a low fluorescence mutant (LF-1) of *Scenedesmus obliquus*

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In the LF-1 mutant of *Scenedesmus obliquus*, a failure to remove a C-terminal extension from the D1 protein of photosystem II (PS II) is associated with the absence of a water-splitting manganese complex. Treatment of LF-1 thylakoids and PS II-enriched membranes with a Triton X-100 extract of wild-type thylakoids results in a specific reduction in molecular mass of the LF-1 D1 to the same value as that in wild-type membranes. Water-splitting activity can be photogenerated in these extract-treated LF-1 PS II-enriched membranes, and in PS II membranes from dark-grown wild-type cells, but not in untreated LF-1 membranes. The results indicate that LF-1 cells lack the D1 processing protease, and that the presence of the D1 extension in LF-1 is directly responsible for preventing assembly of the manganese complex.

Photoactivation; Carboxyl-terminal processing; D1 polypeptide; Photosystem II; (*Scenedesmus obliquus*)

1. INTRODUCTION

It is now generally accepted that the primary photoreactants of PS II are bound to a heterodimer of the D1 and D2 polypeptides [1-5]. Additionally, one or both of these polypeptides have been implicated in binding the tetranuclear manganese cluster [6-9] involved in water oxidation. The extrinsic 33, 23 and 17 kDa proteins of PS II bind to the lumenal side of the complex and appear to optimise the rate of oxygen evolution by stabilising the manganese cluster. Although the role of some of the PS II polypeptides is becoming apparent, the mechanism of assembly and activation of the PS II complex remains relatively unclear. Recently, conditions have been established for photoligation of Mn^{2+} and photoactivation of PS II-enriched membranes from wheat depleted of Mn and extrinsic proteins by chemical treatments [10]. PS II photochemistry appears to be necessary for the oxidation and ligation of Mn^{2+} and activation of the complex into an oxygen-evolving moiety. For optimal activation, Ca^{2+} and the 33 kDa extrinsic protein are required, although the latter is apparently not essential [10]. Another process that occurs during the assembly of the PS II complex is processing of the D1 polypeptide by removal of a 1.5 kDa extension [11-13] thought to be at the C-terminus of the D1 precursor [14]. The mechanism of processing and the function, if any, of this C-terminal extension are unclear.

Recently a number of features of the phenotype of a low fluorescence mutant of the green alga *Scenedesmus obliquus* (LF-1) have been described [15-20]. It appears that the D1 precursor polypeptide in this mutant is not processed but is still assembled into a PS II reaction centre complex

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Abbreviations: PS II, photosystem II; LF-, low fluorescence; C-terminus, carboxyl terminus of protein; N-terminus, amino terminus of protein; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; PBQ, phenyl-*p*-benzoquinone; DCPIP, 2,6-dichlorophenolindophenol

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[18–20]. The mutant thylakoid membranes have less than half the manganese content of wild-type membranes and do not evolve oxygen although electron transport through PS II is possible when artificial electron donors are added [15]. It has therefore been suggested that the presence of the C-terminal extension on the D1 polypeptide of PS II reaction centres from LF-1 in some way prevents the correct assembly of the manganese complex [19,20]. The failure to process D1 may result from either a lack of the processing protease or a failure of the protease to recognise the cleavage sequence in the mutant.

Here, we show that PS II-enriched membranes from wild-type *Scenedesmus*, grown and prepared strictly in the dark, do not evolve oxygen until photoactivation has taken place. Under the same conditions it was not possible to photoactivate PS II-enriched membranes from the LF-1 mutant. Incubation of thylakoids or PS II-enriched membranes from LF-1 with a Triton X-100-solubilised extract from wild-type thylakoids resulted in a change in apparent molecular mass of LF-1 D1 from 34 to 32 kDa. We show that pretreatment of the LF-1 PS II membranes with the extract enables photoactivation of LF-1 to take place.

2. MATERIALS AND METHODS

The wild-type and LF-1 mutant of *S. obliquus* (obtained from Dr N. Bishop, Oregon State University) were grown heterotrophically in the dark on an enriched medium [21]. Thylakoids and PS II-enriched membrane were prepared as described [22]. The D1 polypeptide was labelled in vivo with [^{35}S]methionine (Amersham) by an adaptation [20] of the procedure described in [23].

A Triton X-100-solubilised extract was prepared from wild-type thylakoids as described by Kirwin et al. [24] from 2 l of cell culture grown under illumination (15 $W \cdot m^{-2}$). The thylakoids were washed twice with 10 mM Tricine-NaOH (pH 7.0), 300 mM sucrose, 5 mM $MgCl_2$. The pellet was resuspended in 50 mM Tricine-NaOH (pH 7.0), 15 mM NaCl, 5 mM $MgCl_2$ at a final concentration of 1 mg/ml chlorophyll. Triton X-100 (20%) was added to give a ratio of 2.5 mg Triton/mg chlorophyll. The mixture was stirred at 4°C in the dark for 30 min and then centrifuged at 30000 × *g* for 30 min. The supernatant was retained.

The Triton extract was added as indicated in the figure legends to ^{35}S-labelled LF-1 and wild-type thylakoids suspended in 50 mM Mes-NaOH (pH 6.5), 0.4 M sucrose, 10 mM NaCl (buffer A) at 1 mg/ml chlorophyll. The samples were then incubated in the dark at 25°C as indicated and then prepared for electrophoresis.

For the photoactivation experiments on treated PS II membranes, the Triton extract was pretreated by exposure to high light intensities (≥ 200 $W \cdot m^{-2}$) for 2 h at 4°C. This photoinhibitory treatment removed all residual O_2-evolving activity from the extract. PS II membranes (75 μg chlorophyll) were incubated with 75 μl of the extract for 60 min in the dark at 25°C. The mixture was centrifuged at 30000 × *g* for 10 min and the pellet resuspended in 300 μl buffer A containing 1 mM $MnCl_2$, 50 mM $CaCl_2$ and 100 μM DCPIP. The suspension was exposed to light (5 $W \cdot m^{-2}$) for 45 min. 100 μl was taken for estimation of O_2-evolving activity and the remainder prepared for electrophoresis. Photoactivation of untreated PS II membranes was carried out as in [10]. O_2 evolution was measured at pH 6.5 in a Clark-type O_2-electrode (Hansatech) using PBQ as an electron acceptor.

SDS-PAGE of photosynthetic membranes was performed according to [25] using an acrylamide concentration of 15%. Samples were solubilised in sample buffer prior to loading. Proteins separated by SDS-PAGE were transferred onto nitrocellulose (pore size 0.2 μm). Blots were then probed with a purified antibody to the *lacZ/psbA* gene fusion product (D1-β-galactosidase fusion protein) prepared as in [26] and kindly provided by Dr P. Nixon (Imperial College, London). Bound antibody was visualised with alkaline phosphatase conjugated to goat, anti-rabbit antibodies (Promega). ^{35}S-labelled proteins were visualised by autoradiography of the nitrocellulose blots.

3. RESULTS

Fig.1 shows the pH profile for photoactivation of PS II-enriched membranes from dark-grown wild-type *S. obliquus*. Cultures were grown and membranes prepared under rigorously controlled dark conditions. Prior to photoactivation, these dark-grown preparations did not evolve oxygen.

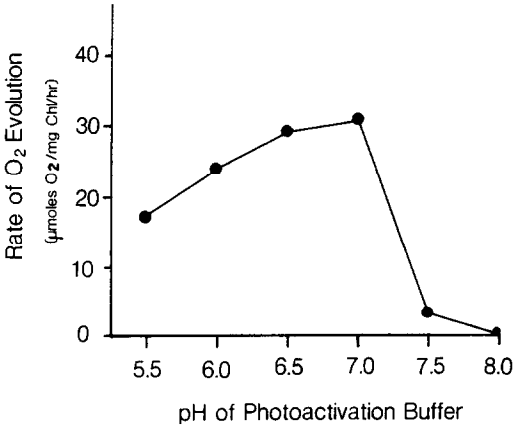

Fig.1. pH profile for photoactivation of PS II-enriched membranes from dark-grown wild-type *S. obliquus*. Photoactivation was carried out as described [10] over the pH range 5.5–8.0, and oxygen evolution was measured at pH 6.5 as described in section 2.

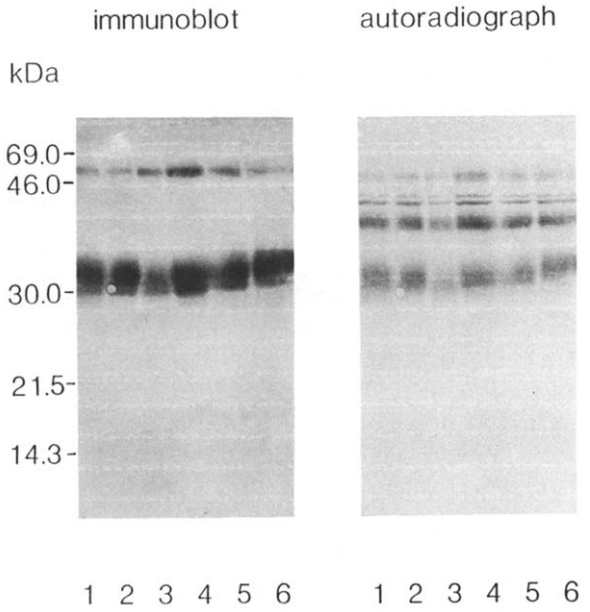

Fig.2. Processing of the D1 polypeptide in thylakoids prepared from the LF-1 mutant of *S. obliquus*. 75 μg Triton X-100 extract prepared as in section 2 was added to 75 μg chlorophyll of [^{35}S]methionine-labelled thylakoid membranes from the wild-type or LF-1 mutant and incubated before the addition of electrophoresis sample buffer as follows. Lanes: (1) wild-type, 2 h; (2) LF-1, 2 h; (3) LF-1, 1 h; (4) LF-1, 30 min; (5) LF-1, 10 min; (6) LF-1, 0 time. (A) Western blot probed with antibodies specific for the D1 polypeptide following SDS-PAGE of the samples. (B) autoradiograph of the immunoblot shown in (A).

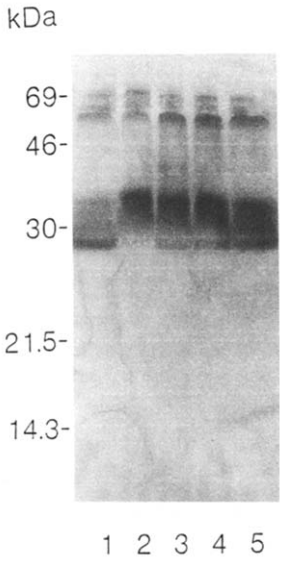

Fig.3. Processing of the D1 polypeptide in PS II-enriched membranes prepared from *S. obliquus*. Membranes were incubated with the Triton X-100 extract prepared from wild-type cells as described in section 2 before being analysed by SDS-PAGE followed by Western blotting with the D1 antibody. Lanes: (1) LF-1 membranes incubated with Triton X-100 extract; (2) untreated LF-1 membranes; (3) untreated membranes from dark-grown wild-type cells; (4) untreated membranes from light-grown wild-type cells; (5) membranes from light-grown wild-type cells incubated with the Triton X-100 extract.

Photoactivation was optimal after 45 min of weak light treatment (5 $W \cdot m^{-2}$) and the pH optimum for the process was 6.5–7.0. The maximum rate of oxygen evolution after photoactivation was 31 μmol O_2/mg chl per h. In PS II membrane preparations from light-grown cultures the O_2-evolution rate was 60 μmol O_2/mg chl per h and hence the maximum photoactivated rate was 52% of the maximum wild-type rate attainable.

A demonstration of the D1 processing activity is shown in fig.2. Intact cells of LF-1 *Scenedesmus* were labelled with [^{35}S]methionine. In thylakoids prepared from these cells the D1 polypeptide is heavily labelled [23]. On incubation of labelled LF-1 thylakoids with a Triton X-100 extract from unlabelled wild-type cells, the D1 polypeptide was reduced in apparent molecular mass by 1–2 kDa from 34 to 32 kDa, the same molecular mass as for D1 from wild-type thylakoids. It is clear that other labelled polypeptides were not affected by the incubation with the Triton X-100 extract. Immunoblotting with an antibody specific for the D1 polypeptide confirmed that the polypeptide that was cleaved by the protease activity in the extract was indeed the D1 polyepeptide. Control experiments emphasise the specificity of the processing; incubation of labelled wild-type thylakoids with the Triton X-100 extract did not result in a change in apparent molecular mass of D1. In all samples analysed by SDS-PAGE the final Triton X-100 concentration was brought to 0.13% before addition of sample buffer. A similar Triton X-100 extract was prepared from both pea and LF-1 thylakoids. Incubation of LF-1 thylakoids with the pea extract resulted in cleavage of D1 to the mature wild-type size, although the activity of this extract was lower than of that prepared from wild-type *Scenedesmus* thylakoids. No processing of LF-1 D1 was observed on incubation with the extract prepared from LF-1 thylakoids.

Processing of D1 in LF-1 was also observed in PS II-enriched membrane preparations. Again treatment of such preparations with the wild-type Triton X-100 extract resulted in a decrease in apparent molecular mass of D1 from 34 to 32 kDa. This shift in molecular mass can be seen in the D1 immunoblot shown in fig.3.

Prior to treatment with the Triton X-100 extract from wild-type cells it was not possible to photoactivate PS II-enriched membranes from LF-1. After the treatment however, photoactivation was possible (table 1). The extent of photoactivation of the LF-1 preparations under optimal conditions was 83% of that observed in PS II preparations from dark-grown wild-type cells.

Preincubation of the dark-grown wild-type PS II membranes with the Triton X-100 extract inhibited the maximum photoactivation to 75% of that observed in the absence of the extract. Photoactivation of PS II-enriched membranes from LF-1 was also possible following treatment with the Triton X-100 extract from pea thylakoids but not after treatment with the extract from LF-1 thylakoids.

4. DISCUSSION

Photoactivation of intact cells of dark-greened algae has previously been reported [27]. In this study we have photoactivated PS II-enriched membranes from dark-greened *Scenedesmus*. Our protocol was essentially the same as that used by Tamura and Cheniae [10] when they photoactivated PS II-enriched membranes from wheat seedlings previously depleted of manganese and the extrinsic proteins by washing with hydroxylamine or Tris. In our experiments however, the membranes were not pretreated to remove endogenous manganese or extrinsic proteins before photoactivation. Although *Scenedesmus* PS II membrane preparations are generally less active than those from higher plants it would appear that the photoactivation process has similar kinetics, ion requirements and pH optimum in the dark-grown algae and hydroxylamine-treated wheat preparations. Without any pretreatment it was not possible to photoactivate PS II-enriched membranes from the LF-1 mutant despite attempts to manipulate the ionic conditions. It is known that the D1 molecule in the LF-1 mutant has a C-terminal extension [19, 20] that is normally removed by a processing protease probably in the stromal lamellae [29]. In all cases studied so far, there is evidence to suggest that C-terminal processing of D1 takes place [11–13,28] although the protease responsible for this has never been characterised. Following processing in the stromal lamellae it is thought that palmitoylation of D1 takes place before migration into the granal lamellae [29]. In LF-1 the PS II reaction centre appears to assemble but the C-terminal extension interrupts the complete assembly of the Mn cluster involved in water oxidation. Here we have shown that cleavage of D1 in the mutant to the same apparent molecular mass as the mature wild-type molecule enables photoligation of manganese and subsequent photoactivation. This result, together with the data of Tamura and Cheniae [10] which indicate that a manganese water-splitting complex can be photoligated in the absence of the extrinsic proteins, suggests that the D1 extension prevents the complete manganese complex from assembling due to direct steric hindrance of the Mn-binding sites rather than to effects on the binding of the extrinsic proteins. Our result also indicates that the lesion in the LF-1 mutant arises from an absence of processing activity rather than a change in the processing site on D1.

The question arises as to the function of the C-terminal extension in the D1 molecule. One possibility is that the extension is required for the correct integration of the D1 polypeptide into the

Table 1

Rate of oxygen evolution of PS II membranes from wild-type and LF-1 cells of *Scenedesmus* before and after treatment with a Triton X-100 extract of wild-type thylakoids for 1 h and weak light treatment for 45 min (see section 2)

	Rate of oxygen evolution (μmol O_2/mg chlorophyll per h)
Light-grown wild-type	60
Dark-grown wild-type	0
Dark-grown wild-type after weak light treatment	31
Dark-grown wild-type after extract treatment and weak light treatment	22.4
Dark-grown LF-1 after weak light treatment	0
Dark-grown LF-1 after extract and weak light treatment	18.6

PS II reaction centre complex, but then needs to be removed before the water-splitting complex can be assembled. Another possibility is that the extension is required to prevent premature binding of manganese, or of other adventitious ions or proteins, until the D1 polypeptide is correctly assembled into the PS II complex.

The only primary lesion identified so far in the LF-1 mutant appears to be the non-processing of D1. A number of other polypeptides involved in photosynthetic electron transport including the '33 kDa' extrinsic protein are also processed by thylakoidal proteases [30]. It has been shown that the 33 kDa protein has an identical molecular mass in both wild-type and LF-1 thylakoids presumably reflecting processing in both cases [31]. This suggests that the protease involved in processing D1 is different from those invoved in N-terminal cleavage. A less likely possibility is that the same protease is involved but the lesion in the LF-1 protease only affects its ability to cleave D1. We are currently characterising and attempting to purify the D1 processing protease.

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