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Assembly of Photosystem II polypeptides and expression of oxygen evolution activity in the chloroplasts of *Euglena gracilis* Z during the dark-light transition

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Euglena **cells (*Euglena gracilis* Z) grown heterotrophically in the dark were illuminated, and the assembly of the polypeptides of Photosystem II in the thylakoid membrane during greening of the cells was studied by sodium dodecylsulfate polyacrylamide gel electrophoresis and Western-blotting analysis with the antibodies against spinach Photosystem II polypeptides. In the dark-grown cells where the chloroplast is not developed, neither chlorophyll nor Photosystem-II-related polypeptide was detected. Upon illumination, chlorophylls and the polypeptides of Photosystem II began to be synthesized and accumulated in the thylakoid membrane. By comparing the amount of these membrane components in greening process of *Euglena* cells, it became evident that the extrinsic 30 kDa protein responsible for stabilization of Mn in oxygen-evolution system accumulated more slowly than the other components comprising the core complex of Photosystem II. In accordance with this, electron transport activity from water to 2,6-dichloroindophenol in chloroplasts appeared later compared with that from 1,5-diphenylcarbazide to 2,6-dichloroindophenol, and the increase in the former activity was in parallel with the accumulation of the extrinsic 30 kDa protein in the thylakoid. These results suggest that accumulation and binding of the extrinsic 30 kDa protein to the core complex of Photosystem II is rate-limiting in the light-induced organization of Photosystem II and expression of the oxygen-evolution activity in *Euglena*. This feature is in contrast with that of higher plants and algae, where a considerable amount of extrinsic proteins of Photosystem II already exists in the dark growth condition and accumulation of the extrinsic proteins in the membrane itself is not a major determinant of the expression of oxygen evolution activity.**

Introduction

PS II participates in oxidation of water molecules to evolve oxygen in chloroplasts [1,2]. Several membrane components have been shown to be indispensable for this process. The reaction center components of PS II, i.e., a specific form of chlorophyll *a* as the primary electron donor and a pheophytin *a* as the primary electron acceptor, are located at the so-called D1 and D2 polypeptides having relative molecular mass of 30000–34000 [3,4]. The secondary electron donor Z, the first stable electron acceptor, Q_A, and the secondary acceptor, Q_B, are also bound to these polypeptides. Photochemical reaction at the reaction center produces a strong oxidant, a chlorophyll *a* cation radical, and the oxidizing equivalents produced in this process are transferred through Z to Mn atoms in PS II and are accumulated in them. The Mn atoms probably form a specific cluster structure and oxidize the coordinated water molecules to produce oxygen [5,6]. The other essential components for oxygen evolution are the extrinsic polypeptides of PS II [7–9]. With the spinach PS II particles having a high oxygen evolution activity, three polypeptides of PS II were identified [8]. They have relative molecular masses of 33000, 24000 and

Abbreviations: PS II, Photosystem II; D1 and D2 proteins, proteins carrying the reaction center components of PS II; Z, the secondary electron donor of PS II; Q_A, the first stable electron acceptor of PS II; Q_B, the secondary electron acceptor of PS II; OEC I, II and III, the extrinsic proteins of PS II having relative molecular masses of 33000, 24000 and 18000; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; K-P sorbitol, a solution containing 50 mM potassium phosphate (pH 7.0) and 0.33 M sorbitol; DCIP, 2,6-dichloroindophenol; Mes, 4-morpholineethanesulfonic acid; CP43 and CP47, the antenna chlorophyll-protein complexes having relative molecular masses of 43000 and 47000; LHC, light-harvesting chlorophyll-protein complex; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl) ethyl]glycine; DPC, 1,5-diphenylcarbazide.

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18000 (hereafter these polypeptides are referred to as OEC I, II and III in order of decreasing relative molecular mass), and one of them (OEC I) was shown to be crucial for the stabilization of Mn atoms in the oxygen evolution system [10,11].

Analysis of biogenesis of PS II is one of the promising ways for elucidating the strucure of PS II. During the development of PS II in chloroplasts, the constituents of PS II have to be synthesized and organized properly in the thylakoid membrane to enable an efficient photochemical reaction. So far, the process of asembly of the PS II components in the thylakoids membrane has not been studied extensively, and information on this problem is quite limited. In the present study, we investigated the assembly of the PS II components in greening process of *Euglena* cells mainly with SDS-PAGE and Western blotting analysis. The results suggest that the accumulation of OEC I in the thylakoid primarily determines the organization of the oxygen evolution system and expression of its activity in *Euglena*.

Materials and Methods

Dark-grown cells of *Euglena gracilis* Z were maintained in a stock medium (Hutner's heterotrophic medium [12]) at 25°C. For dark-light transition of the cells, a small amount of the dark-adapted cells were inoculated in a fresh Hutner's medium (3 liter) and cultured in the dark for about 1 week. The cells reached to early stationary growth phase were illuminated by fluorescent lamps (1500 lx). 100-ml aliquots of the culture were withdrawn from the total culture after given times of illumination and the number of cells was counted microscopically with a hemacytometer. The cells were collected by centrifugation at $5000 \times g$ for 3 min, and washed once with a medium containing 50 mM potassium phosphate (pH 7.0) and 0.33 M sorbitol (K-P sorbitol).

For measurement of DCIP photoreduction, the cells were briefly sonicated (20 kHz, 30 s) and centrifuged at $144000 \times g$ for 10 min. The pellet was washed once with K-P sorbitol and used as broken chloroplasts. DCIP photoreduction was measured with a Hitachi 356 dual-wavelength double-beam spectrophotometer. A xenon lamp was used as the actinic light source and red actinic light was obtained by the use of a VR 67 filter (Toshiba). A KL-58 interference filter (Toshiba) and a 4-96 filter (Corning) were used in front of the photomultiplier. The wavelength of the measuring beam was 580 nm and the slit width was 6 μm. The reaction mixture (1 ml) contained 100 mM Mes (pH 6.5) and broken chloroplasts which were obtained from $4 \cdot 10^5$ *Euglena* cells grown under the given periods of illumination.

For the assay of chlorophyll and carotenoid, *Euglena* cells were extracted thrice with 80% acetone and the combined extracts were analyzed spectrophotometrically with a Hitachi 557 spectrophotometer.

SDS-PAGE and Western-blotting were done as previously described [13]. The whole cells were solubilized for SDS-PAGE, and the number of cells put in each lane of the gel was adjusted to $4 \cdot 10^5$. The amount of protein was determined by the method of Lowry et al. [14]. Preparation of the antibodies against the extrinsic 33 kDa protein, the reaction-center-binding D1, D2 proteins, the apoproteins of CP43 and CP47, and the apoprotein of LHC has been described previously [13,15]. The protein bands in the nitrocellulose membranes in Western-blotting were analyzed by a Shimazu dual-wavelength TLC scanner CS-930. The density of the proteins on the nitrocellulose membranes developed by the reaction of peroxidase-conjugated antirabbit goat IgG, peroxide and reduced 4-chloro-1-naphthol was measured at 510 nm. Before the quantitation of the density, a linear relationship between the amount of protein and the density was ascertained.

For identification of the D1 protein in *Euglena*, intact chloroplasts were isolated and in organello translation was carried out. Isolation of intact chloroplast was done according to the method of Price and Cushman from vitamin-B-12-sufficient cells [16]. The intact chloroplasts were incubated with a solution containing 50 mM Tricine-KOH (pH 8.4), 0.33 M sorbitol and 10 μCi [^{35}S]methionine (New England Nuclear) at 25°C and illuminated by a tungsten lamp (10000 lx) for 20 min. After centrifugation at $20000 \times g$ for 3 min, a solution containing 63 mM Tris-HCl (pH 6.8), 10% glycerol, 5% β-mercaptoethanol and 2.3% SDS was added to the pellet to stop the synthesis of protein. The pellet was analyzed by SDS-PAGE, where a 15% polyacrylamide gel containing 6 M urea was used as a resolving gel. The gel was stained with Coomassie brilliant blue and treated with Enlightning (New England Nuclear) according to the manufacturer's manual. The gel dried was brought into contact with a X-ray film (Fuji), and stored at -80°C for 2 days. The film was developed and a fluorogram was obtained. Other details of the experimental procedures are shown in the legend of each figure.

Results and Discussion

In the dark-grown *Euglena* cells, chloroplasts are not developed and remain as proplastids. Chloroplasts are formed reversibly on illumination of the cells, and strict regulation by light seems to exist both in organizing the photosynthetic apparatus and expression of the photosynthetic activities [17]. To see the effect of light on the organization of PS II in *Euglena*, we first carried out SDS-PAGE for the whole proteins of the dark- and

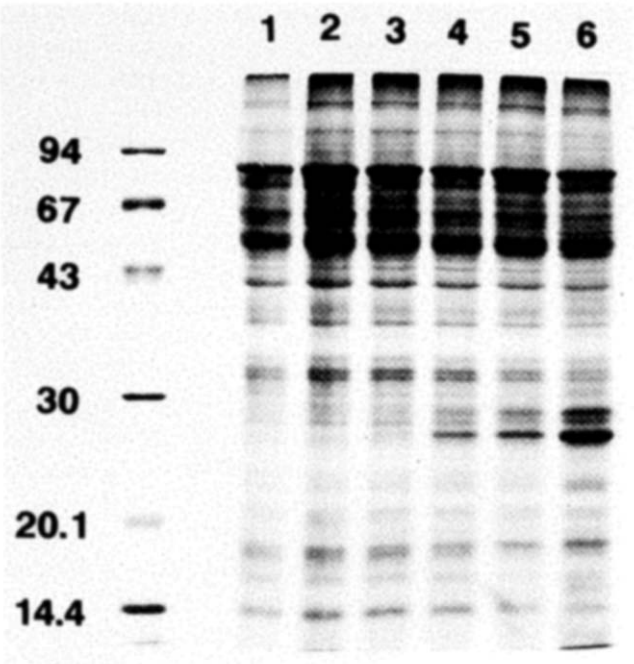

Fig. 1. A SDS-polyacrylamide gel showing the total proteins of *Euglena* cells in the greening process. The far left lane is the molecular weight markers. Lane 1: the dark-grown cells. Lanes 2–6: the cells illuminated by white fluorescent lamps (1500 lx) for 8, 17, 26, 29 and 55 h, respectively, after dark incubation. The cell number in each lane was $4 \cdot 10^5$.

light-grown *Euglena* cells, and compared their polypeptide profiles with each other (Fig. 1). The amount and number of protein detected in the dark-grown cells were less than those of the light-grown cells. Especially, the amounts of the proteins ranging from 20 to 40 kDa were low in the dark-grown cells, which in turn greatly increased by the illumination. The most prominent protein appeared after the illumination of the cells was the apoprotein of LHC, which has a relative molecular mass of about 27000. It was observed that chlorophyll-protein complexes other than LHC, e.g., CP43 and CP47 of PS II, in *Euglena* were quite unstable and easily dissociated into free chlorophylls and apoproteins in the conventional SDS-PAGE. The apoprotein of LHC, however, binds chlorophylls stably in *Euglena* and was discerned easily in the electrophoresis gel. Many other proteins appearing under the illumination were not identified here, but a considerable difference was noticed in the rate of accumulation among these proteins.

In the dark, carotenoid was the predominant pigment in the cells. There was a significant increase in the amount of chlorophyll in parallel with the appearance of the specific proteins in the light, reflecting the development of chloroplasts (Figs. 2 and 3). Chlorophyll *a* to *b* ratio in the greening cells may become one of the measures for the formation of LHC. LHC is the most abundant chlorophyll-protein complex in the thylakoid membrane, and in contrast to the other antenna chlorophyll complex, it abounds in chlorophyll *b*. Chlorophyll *a* to *b* ratio in the cells, therefore, should come close to that of LHC as LHC is accumulated in the thylakoid as the major component. In the earliest stage of greening in *Euglena* cells, the content of chlorophyll *a* prevailed over that of chlorophyll *b*, and as the greening steps proceeded, the chlorophyll *a* to *b* ratio settled down to a value of about 2 (Fig. 3). These results suggest that the formation of the reaction center chlorophyll *a* and the antenna chlorophyll *a* species precedes the synthesis of the bulk light-harvesting chlorophyll *a* and *b* species of LHC.

Synthesis of the protein components of PS II in the greening process was followed by SDS-PAGE and Western-blotting analysis with whole cells of *Euglena* (Fig. 4). As D1 and D2 proteins have significant homology in their amino-acid sequences [19], and as some

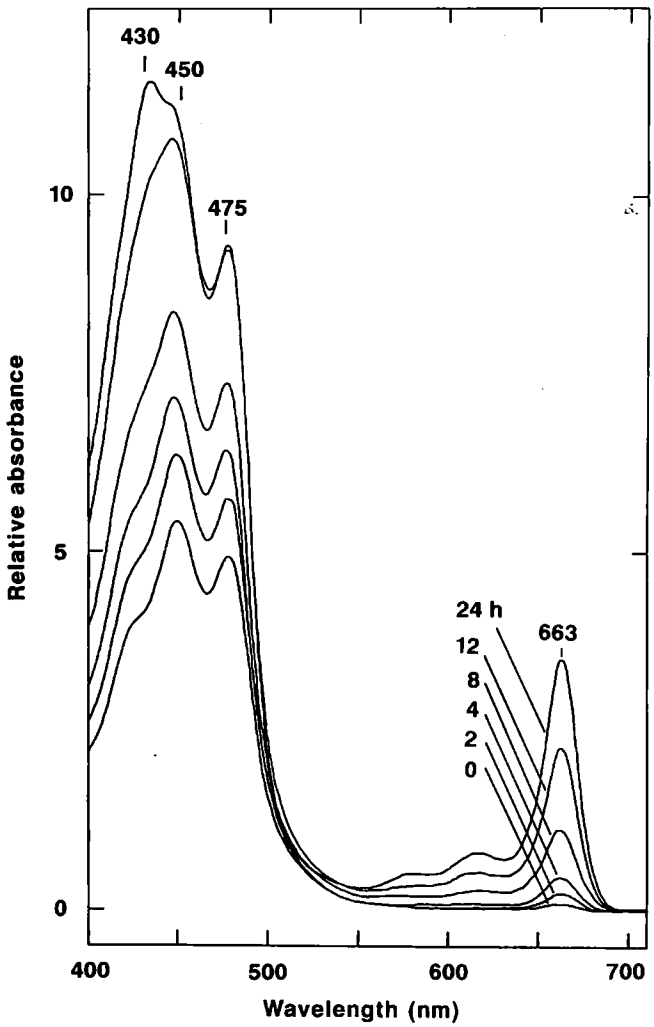

Fig. 2. Absorption spectra of the acetone extract of *Euglena* cells. The cells grown in the dark or illuminated for given periods were extracted three times by 80% acetone. The extracts were combined and the absorption spectra were measured. The number of cell used for each extraction was $2.6 \cdot 10^5$.

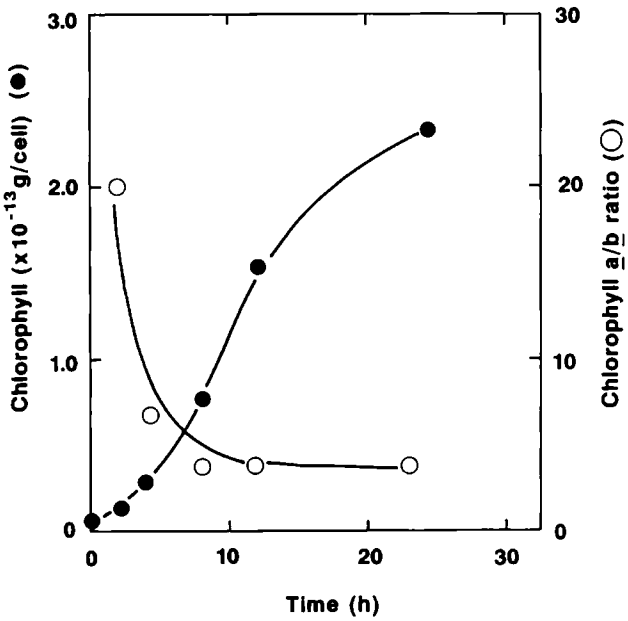

Fig. 3. Amount of chlorophyll and chlorophyll *a*/*b* ratio in *Euglena* cells in the greening process.

degradation products of D1 and D2 proteins may be present in the cells, it is expected that the antibody against D1 protein used here will cross-react with several protein components in the gel. The aggregates of the D1 and D2 proteins are also responsible for the multiple cross-reaction bands in the immunoblotting analysis. To identify the D1 protein band in a more convincing way in the gel, we isolated intact chloroplasts from *Euglena* cells and carried out in organello translation with methionine labeled with ^{35}S. The labeled methionine is incorporated into chloroplasts and distributed in the rapidly synthesized proteins in chloroplasts. As the D1 protein is known as the major protein which appears after a short illumination of chloroplast in vitro [20], it is possible to identify the protein by radiolabeling under illumination and subsequent SDS-PAGE and fluorography analysis. The fluorogram showed that the protein having M_r of 28000–30000 is the major protein incorporating the labeled methionine, depending on illumination (Fig. 5). Several other proteins were labeled weakly during the dark incubation period of 20 min. From the analysis of DNA sequence in the *psbA* gene in the chloroplast, it was shown that precursor of the D1 protein in *Euglena* contains 345 amino acids compared with 353 in spinach D1 protein and has a calculated M_r of 38321 [18]. Probably there are several processing steps in the maturation of *Euglena* D1 protein.

As the 28–30 kDa protein is assignable to the D1 protein, the density of the corresponding band in Western-blotting with the antibody against the D1 protein was plotted against the illumination time of *Euglena* cells (Fig. 6). The relative values are shown with the value obtained from the fully greened cell as the 100%

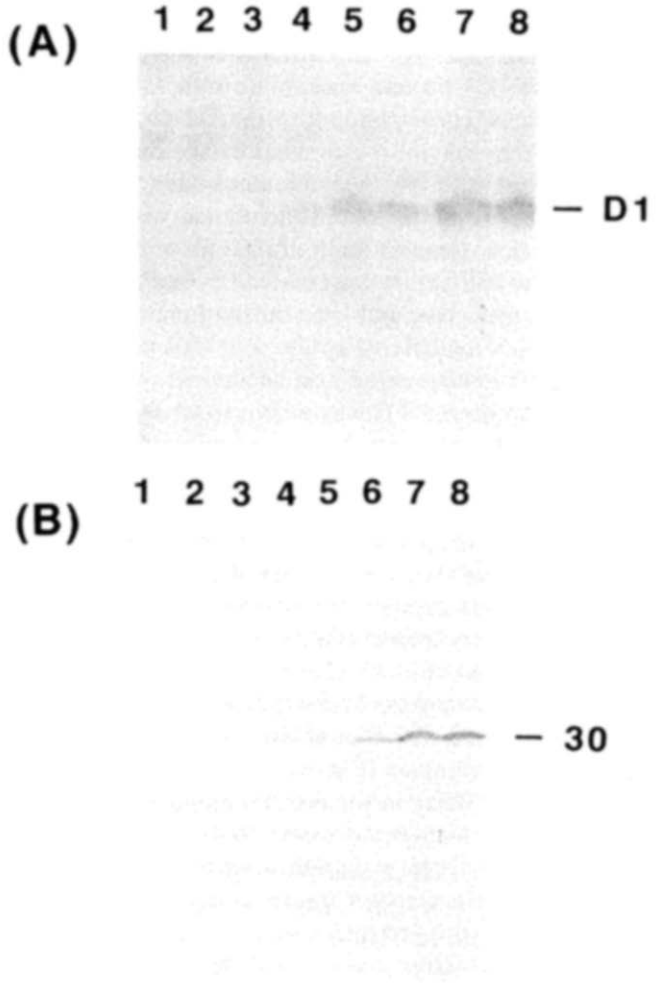

Fig. 4. Western blotting analysis of the proteins in *Euglena* cells showing D1 and the extrinsic 30 kDa protein. (A) D1 protein. (B) The 30 kDa protein. Lane 1: the dark-grown cells. Lanes 2–8: the cells were illuminated for 2, 4, 8, 12, 24, 48 and 72 h, respectively, after dark incubation. The number of cells introduced into each lane was the same as that of Fig. 1.

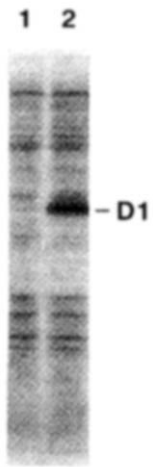

Fig. 5. A fluorogram showing the rapidly synthesized protein in intact chloroplasts of *Euglena* under illumination. Lane 1: intact chloroplasts incubated with [^{35}S]methionine for 20 min at 25 °C in the dark. Lane 2: intact chloroplasts incubated as lane 1 but under illumination with white light (10000 lx).

standard. Under the illumination conditions employed here (1500 lx), the half-saturation time for the accumulation of the D1 protein was about 10 h. The amounts of the proteins corresponding to the D2 protein, CP43, CP47 proteins were also estimated by the immuno-blotting analysis with the specific antibodies. They also accumulated in the thylakoid membrane with the same half-saturation time as that of D1 protein (data not shown). The half-saturation times of these PS II components were also comparable to those of chlorophyll and the apoprotein of LHC (Fig. 3).

The antibody prepared against the extrinsic 33 kDa protein in spinach PS II was shown to crossreact with a protein having an apparent molecular mass of 30 kDa in *Euglena*. The spinach 33 kDa protein has been extensively characterized and has been shown to play a crucial role in oxygen evolution by stabilizing the Mn atoms in the catalytic site. In the dark-grown *Euglena* cells, no 30 kDa protein was detected by the immunoblotting analysis. After transition of the dark-grown cells to the light, the 30 kDa protein appeared, but a considerable delay was observed in accumulation of the protein compared with that of chlorophyll and the other PS II core components (Fig. 6).

The specific delay in the accumulation of the 30 kDa protein in the membrane seems to be critical for the expression of oxygen evolution activity in chloroplasts. By measuring the electron transport activity of *Euglena* chloroplasts in the greening process, it was shown that DCIP photoreduction activity with H_2O as an electron donor developed later compared with that with DPC as a donor. Apparently, the former activity appeared in parallel with the accumulation of the 30 kDa protein in the membrane (Fig. 7).

These results suggest the following scheme for the assembly of the PS II complex in *Euglena*. Illumination of the dark-grown cells induces synthesis of the

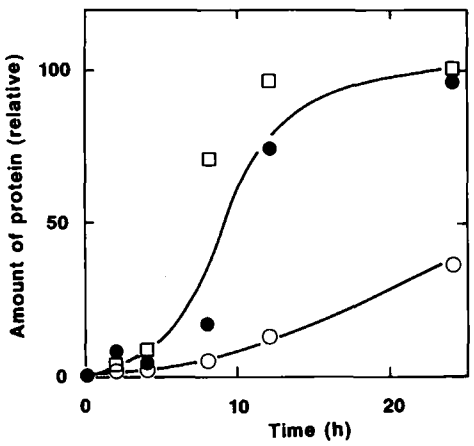

Fig. 6. The amount of PS II proteins accumulated in *Euglena* cells in the greening process. ●, D1 protein. ○, the extrinsic 30 kDa protein. □, apoprotein of LHC. The amount of each protein in $4 \cdot 10^5$ cells is shown.

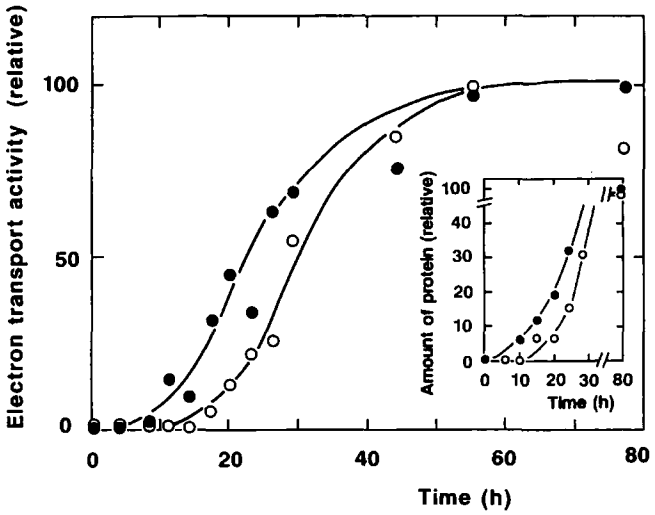

Fig. 7. Electron transport activity and accumulation of PS II proteins in thylakoid during the greening process of *Euglena* cells. ●, DCIP photoreduction with DPC as an electron donor. ○, DCIP photoreduction with water as an electron donor. In the inset, closed and open circles show the amounts of LHC apoprotein and the extrinsic 30 kDa protein in the thylakoid, respectively.

apoproteins of the core components in PS II, i.e., D1, D2, CP43, CP47 and probably cytochrome *b*-559, and they are rapidly incorporated into the thylakoid membrane. Chlorophylls in the reaction center and antenna of PS II may also be synthesized at the same stage, and the formation of the functional unit of the PS II reaction center complex may be completed by their binding to the apoproteins. All these components are known to be encoded by chloroplast DNA [21]. The extrinsic 30 kDa protein and apoprotein of LHC are encoded by nuclear DNA [22,23] and thus it is possible that their expression is regulated differently from that of the core components [24]. These nuclear encoded proteins are synthesized in the cytoplasm and their precursors are transported through chloroplast envelope membrane [25]. In the case of the 30 kDa protein it must cross the thylakoid membrane also. In the earlier stage of development of *Euglena*, protein transport system is probably not fully functional, which may be a cause for the delay of the assembly of the specific proteins. In that case, expression of ATPase activity and the specific enzymes responsible for the processing may limit the assembly of the proteins in chloroplasts. Transport of the 30 kDa protein across the envelope and thylakoid membranes should be most affected under these conditions.

It is possible that both the membrane-bound and free 30 kDa proteins are present in the cell, depending on the growth stage. The membrane-bound protein is involved in oxygen evolution process directly and the free protein is reserved in the lumenal space of thylakoid for replacement. As we used the whole cells as the material for the assay of protein accumulation, both the mem-

brane-bound and free 30 kDa proteins may be detected here. However, as the 30 kDa protein exists only as a membrane-bound form in the fully-greened cells (data not shown), there seems to be strict regulation to coordinate the production of the extrinsic 30 kDa protein with that of the intrinsic proteins of PS II in the developing process of PS II, and no excess 30 kDa protein is produced in chloroplasts.

The feature of PS II assembly in *Euglena* is different from that reported with other higher plants and algae. In maize, the extrinsic 33 kDa protein is present in the chloroplast of the dark-grown plant and its content increases upon illumination [26]. As there is no core component of PS II and LHC in the dark-grown maize, their synthesis seems to be rate-limiting in the expression of oxygen evolution. In relation to the synthesis of proteins in PS II in maize, it was reported that the level of the extrinsic 33 kDa protein in the thylakoid membrane is mediated in part by phytochrome, whereas accumulation of LHC is dependent on chlorophyll synthesis [26]. Delay of appearance of LHC in the membrane after illumination compared with the 33 kDa protein in maize may be attributable to the different activation of the photoreceptors regulating the protein synthesis under the illumination condition employed. In *Euglena*, participation of phytochrome in chloroplast is not yet ascertained [17], and there is a possibility that induction of the 30 kDa protein depends on accumulation of a certain level of chlorophyll. The presence of a considerable amount of the 33 kDa protein in the dark-grown plant was also reported with barley [27]. In green alga *Chlamydomonas reinhardtii*, the cells have chloroplasts in the dark and all the PS II components, including the core and extrinsic proteins, are detectable [28]. In *Chlamydomonas*, expression of oxygen evolution activity is determined by a factor other than the accumulation of the related membrane components in chloroplasts. Probably incorporation of these components in their specific functional sites in the membrane under the light is requisite for the development of oxygen evolution activity in this organism. A similar situation applies to a gymnosperm spruce [29].

Euglena has only tiny proplastides in the cell in the dark growth conditions. That is in contrast with the plant and algal species described above which contain immature but large plastids in the dark. In addition to the lack of protein transport system, there may be rather significant limit of volume in *Euglena* proplastide which does not allow for storage of excess proteins. The extrinsic protein may be incorporated into the proplastide only after the intrinsic proteins of PS II begin to be assembled by illumination, which is in parallel with maturation of chloroplast.

In the present studies, we showed accumulation of the extrinsic 30 kDa protein in PS II of *Euglena*. The proteins corresponding to the 24 and 18 kDa protein of spinach PS II were barely detected in the thylakoid of *Euglena* by the Western-blotting analysis carried out here (data not shown). If these two proteins are deleted in *Euglena* chloroplasts, the extrinsic 30 kDa protein should have a determinative role in oxygen evolution activity as the exclusive extrinsic protein in this organism. As the 30 kDa protein is shown to be responsible for stabilization of Mn atoms in the catalytic center of oxygen evolution system, coordination of Mn atoms to the appropriate protein and formation of a stable cluster structure of Mn atoms in the presence of the 30 kDa protein may be required for the expression of oxygen evolution activity in *Euglena* chloroplasts. Light probably plays some crucial role in the ligation of Mn to PS II complex and that should be also elucidated in the greening process. Under the present condition, *Euglena* cells were grown in an enriched culture medium and Mn is not limiting the growth of the cells. Further analysis of the organization of oxygen evolution system has to be carried out in an Mn-limited growth condition to reveal the role of Mn in the assembly of PS II polypeptides and expression of oxygen evolution activity.

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