S8-025

Degradation of OEC33 during strong illumination of the PS II: possible participation of reactive oxygen species generated at the lumenal side of PS II

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Keywords: PS II, OEC33, reactive oxygen species

Inroduction

During the photoinhibition of PS II, the OEC subunits are released from PS II following the degradation of the D1 protein. The OEC subunits re-assemble in PS II after the repair of the photodamaged D1. The OEC33 is stable and has a long life. So far, no specific protease has been reported for the OEC33.
In the present study, we show degradation of the OEC33 during the photoinhibition of PS II. Participation of hydroxyl radicals generated at the lumenal side of PS II is suggested in the degradation of the OEC33.

Materials and Methods

Spinach PS II membranes were suspended in a solution containing 0.4 M sucrose, 10 mM NaCl and 40 mM MES-NaOH (pH 6.5) at 0.5 mg chlorophyll ml^{-1}. After photoinhibition by strong light (4,000 µE m^{-2} s^{-1}), the samples were centrifuged at 16,000 *g* to separate the membrane and soluble fractions. The scavengers of reactive oxygen species, i.e., histidine (5 mM), DABCO (2 mM), mannitol (20 mM), catalase (0.1 mg ml^{-1}), SOD (50 U ml^{-1}), Cyt *c* (80 µM) and Tiron (2 mM), were added to the samples, where indicated. EDTA and EGTA were used at the concentrations of 3-10 mM. Intact thylakoids were prepared as described previously (Ferjani et al. 2001), and suspended in a solution contains 0.4 M sorbitol, 10 mM NaCl, 5 mM $MgCl_2$ and 50 mM Tricine-KOH (pH 7.6). After photoinhibitory illumination, the thylakoids were treated with Triton X-100 (0.1%), incubated on ice for 3 min, and centrifuged at 100,000 *g* for 8 min. The supernatants were centrifuged again at 200,000 *g* for 15 min, and the final supernatants were used as the lumen fractions. SDS/urea-PAGE, Western blot analysis and fluorography were carried out as described previously (Ferjani et al. 2001). Determination of the proteins was carried out by a Personal Scanning Imager PD110 (Molecular Dynamics, U.S.A.). The OEC33 was isolated as described previously (Yamamoto et al. 1998). Where indicated, the OEC33 and the PS II membranes were treated with either H_2O_2 (5-50 mM) or H_2O_2 (0.2-5 mM) plus 1 mM $FeSO_4$.

Results

The effects of strong light on the OEC33 First, we determined the OEC33 released from PS II and that retained in PS II after strong illumination of the PS II membranes (Fig.1). Apparently the amount of the OEC33 detected in the supernatant of the illuminated PS II membranes was smaller than that lost from the PS II membranes. The OEC33 released from

the PS II membranes shows a diffused band in the fluorogram (data not shown), suggesting that the protein is damaged. No such changes were observed in the OEC33 retained in PS II after the illumination (data not shown).

The effects of the scavengers of reactive oxygen species on the light-induced damage to the OEC33 The reactive oxygen species generated in PS II may damage the OEC33 during the strong illumination. Actually, the scavengers of superoxide, such as SOD, Cyt *c* and Tiron, suppressed the damage to the OEC33 (Fig.2). Chelating agents, such as EDTA and EGTA, also suppressed the damage (data not shown). These results strongly support the view that reactive oxygen molecules damage the OEC33.

The effects of externally added reactive oxygen species on the membrane-bound and free OEC33 The OEC33 bound to the PS II membranes and the free OEC33 were treated by externally added reactive oxygen species to see which form of the OEC33 is susceptible to the oxygen radicals. Hydroxyl radicals produced by the reaction of H_2O_2 and $FeSO_4$ damaged the membrane-bound OEC33 and caused a diffused band of OEC33 in the fluorogram (Fig.3). The free OEC33 disappeared by the treatment with hydroxyl radicals, although no

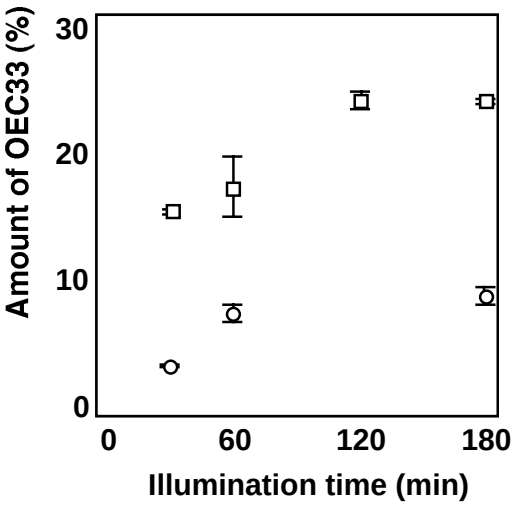

Fig.1. Quantification of the OEC33 in PS II during strong illumination. PS II membranes were illuminated with strong light (4,000 μE m^{-2} s^{-1}) for the time periods indicated and then centrifuged to separate the membrane and soluble fractions. The samples were subjected to SDS/urea-PAGE and Western blot analysis with specific antibody against the OEC33, and then the amount of the protein in each fraction was determined by densitometric analysis of the fluorogram. Circles, the OEC33 detected in the supernatant. Squares, the OEC33 lost from the PS II membranes. The data are means ±S.D.

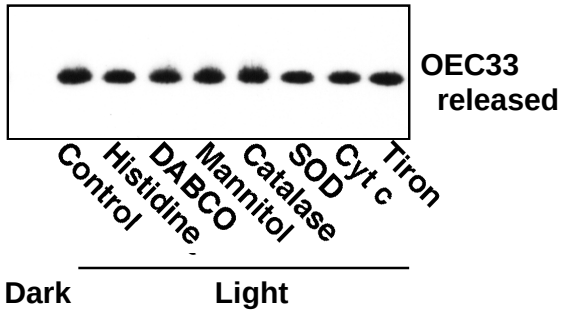

Fig.2. The effects of the scavengers of reactive oxygen species on the light-induced damage of the OEC33. PS II membranes were illuminated with strong light (4,000 μE m^{-2} s^{-1}) for 180 min and then centrifuged. The supernatant was subjected to SDS/urea-PAGE and Western blot analysis with specific antibody against the OEC33. The scavenger added to the sample prior to illumination is indicated below each lane.

degradation fragments or aggregation products were detected (Fig.3). By contrast, H_2O_2 had no effects on both the membrane-bound OEC33 and free OEC33 (Fig.3).

The effects of strong light on intact thylakoids Release of the OEC33 and damage to the free OEC33 were also observed when intact thylakoids were illuminated with strong light (data not shown). In contrast to the case of PS II membranes, however, the externally added scavengers of superoxide and chelating agents did not suppress the damage to the OEC33 (data not shown). These results suggest that the reactive oxygen species, which damage the OEC33, are generated at the lumenal side of PS II during strong illumination. The electron donors to PS II inhibited the damage to the OEC33 in the PS II membranes during the strong illumination (data not shown).

Discussion

Of the turnover process of the OEC33, its synthesis, transport and assembly have been well characterized, while the degradation of the protein is poorly understood. The OEC33 is a very stable protein, and the degradation of the protein has not been demonstrated clearly. In the present study, we found the decrease in the amount of the OEC33 during strong illumination

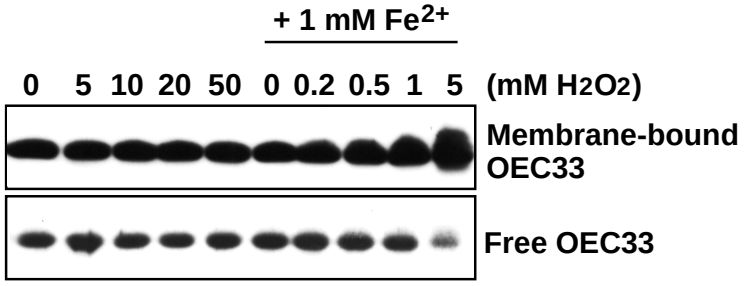

Fig.3. The effects of externally added H_2O_2 and hydroxyl radicals on the membrane-bound and free OEC33. PS II membranes and isolated OEC33 were incubated with H_2O_2 for 10 min in darkness. The reaction was stopped by the addition of catalase. To generate hydroxyl radicals, 1 mM $FeSO_4$ was added. The OEC33 was detected by the method described in the legend to Fig.1.

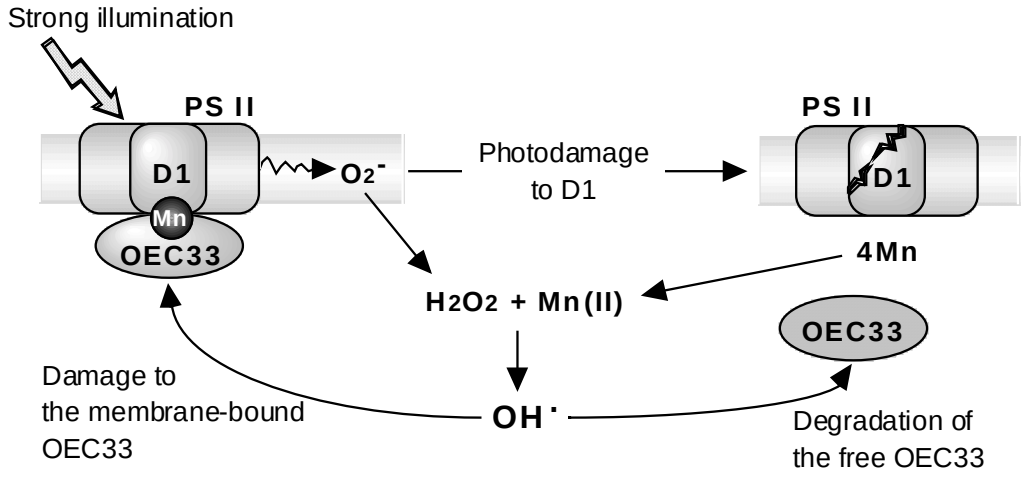

Fig.4. A model showing the degradation of the OEC33 during photoinhibition. Superoxide radicals are generated in PS II during strong illumination, which then generate hydrogen peroxide by disproportionation of superoxide. Hydroxyl radicals are produced by the reaction with the hydrogen peroxide and Mn (II) derived from the Mn cluster. The hydroxyl radicals damage the membrane-bound OEC33. The hydroxyl radicals also induce damage and degradation of the free OEC33.

(Fig.1). Hydroxyl radicals are the most probable candidate to damage and disintegrate the OEC33 (Figs. 2 and 3). It is well known that the Mn atoms are liberated from PS II when the OEC33 is released by strong light. If H_2O_2 is produced at PS II during the strong illumination, the free Mn (II) may react with H_2O_2 to generate hydroxyl radicals. In accordance with that, the isolated OEC33 is disintegrated when H_2O_2 and Mn (II) are used for generation of hydroxyl radicals (data not shown).
The site of production of the oxygen radicals seems to be at the lumenal side of PS II. We reached this conclusion by examining the effects of scavengers of oxygen radicals in intact thylakoids. Taking account of this, we proposed a model showing generation of hydroxyl radicals and damage to the OEC33 in PS II (Fig.4). Further studies are required to detect and identify the oxygen radicals at the donor-side of PS II.

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