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Saturating irradiance-induced photoinhibition without monomerisation of photosystem 2 dimer in soybean leaves

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Abstract

The oligomeric state of photosystem 2 (PS2) complex in soybean leaves treated with saturating irradiance was studied by non-denaturing polyacrylamide gel electrophoresis (PAGE) and gel filtration chromatography. PS2 dimers resolved by non-denaturing PAGE accounted for about 75 % of total PS2 complex and there was no significant difference in the ratio of PS2 dimer to monomer between samples from saturating irradiance-treated and fully dark-adapted leaves. Furthermore, BBY particles were resolved into four chlorophyll-enriched fractions by gel filtration chromatography. From their molecular masses and protein components, these fractions were deduced to be PS2 dimer, PS2 monomer, oligomeric light-harvesting complex 2 (LHC2), and monomeric LHC2. Also, no change in the proportion of PS2 dimer in total PS2 was observed in the granal region of thylakoid membranes from soybean leaves after saturating irradiation. Hence the dimer is the predominant natural form of PS2 *in vivo* and no monomerisation of PS2 dimer occurs during saturating irradiance-induced photoinhibition in soybean leaves.

Additional key words: dimer; *Glycine max*; light-harvesting complex 2; monomerisation; photosystem 2; proteins.

Introduction

Whether PS2 exists as monomer or dimer *in vivo* is still a matter of debate. Holzenburg *et al.* (1993) analysed the three-dimensional structure of PS2 containing oxygen-evolving complexes and antenna proteins, and concluded that PS2 is monomeric. Such a conclusion is based on the fact that the molecular mass estimation fits with the expected mass of a reaction centre surrounded by the antenna proteins. However, Lyon *et al.* (1993), with a two-dimensional analysis of PS2 crystals lacking both the oxygen-evolving complexes and the antenna proteins, concluded instead, on similar bases, that PS2 is dimeric. Moreover, biochemical studies (Santini *et al.* 1994, Hankamer *et al.* 1997a, Bianchetti *et al.* 1998, Zheleva *et al.* 1998) and single particle analyses of two-dimensional crystals (Rhee *et al.* 1998, Nield *et al.* 2000) suggest that most of PS2s are in the dimeric form *in vivo* in both higher plants and cyanobacteria. Recently, intact and highly active dimeric PS2-LHC2 supra-complexes have been isolated directly from spinach thylakoids (Eshaghi *et al.* 1999) and three-dimensional crystal of PS2 dimer with high oxygen evolution activity (Zouni *et al.* 2001) has been obtained, supporting the idea that the dimer is the natural state of PS2.

The PS2 dimer is active, while the PS2 monomer is inactive (Hankamer *et al.* 1997b). Under strong irradiance, PS2 dimers turn into monomers, then the monomers migrate from the granal region to the stromal region of thylakoid membranes to undergo D1 degradation (Barbato *et al.* 1992, Kruse *et al.* 1997, Gonzalez *et al.* 1999). Also, phosphorylation of PS2 core proteins can prevent monomerisation of the PS2 dimers under strong irradiance stress (Kruse *et al.* 1997). However, the suggestions are challenged by some other studies. Kitmitto *et al.* (1999) examined the changes in the size of single PS2 particle, and argued that photoinhibition does not cause a change in oligomeric form of the PS2 complex. Kim *et al.* (1993) have even suggested that photodamaged PS2 reaction centres aggregate into dimer under

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Abbreviations: BBY, photosystem 2-enriched membranes; Chl, chlorophyll; DM, *n*-dodecyl-β-D-maltoside; FPLC, fast protein liquid chromatography; LHC2, light-harvesting complex 2; OG, octyl-β-D-glucopyranoside; PAGE, polyacrylamide gel electrophoresis; PS2, photosystem 2; SDS, sodium dodecyl sulfate.
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stress. Therefore, it is still uncertain whether the change in oligomeric state of PS2 occurs during photoinhibition.

To explore the relationship between photoinhibition and PS2 oligomeric state, the PS2 complexes from soybean leaves were resolved by non-denaturing PAGE and gel filtration chromatography. Evidence indicates that the PS2 dimer is the predominant form *in vivo*, and no monomerisation occurs during photoinhibition caused by saturating irradiance treatment in soybean leaves.

Materials and methods

Plants of soybean (*Glycine max*) were grown in pots at 25/18 °C (day/night) in a phytotron with a 12/12 h (light/dark) period. The photosynthetic photon flux density (PPFD) of irradiance from four xenon lamps was 250 μmol m^{-2} s^{-1} at the top of the plants. The plants were watered everyday. Experiments were performed using fully expanded leaves.

Irradiance treatment: Soybean plants grown in pots were transferred from the phytotron to a laboratory. Following dark adaptation for 3 h, the leaves were irradiated for 3 h at a PPFD of 700 μmol m^{-2} s^{-1} at which photosynthesis is saturated in the leaves of soybean grown in the phytotron. The actinic irradiance from a halogen lamp passed through a layer of flowing water above the leaves to remove heat. After saturating irradiance treatment, the photochemical efficiency of PS2, F_v/F_m, decreased to about 0.70 *vs.* 0.84 of the dark-adapted leaves. After measurements of chlorophyll (Chl) fluorescence, the soybean leaves were frozen immediately in liquid nitrogen for further use.

Isolation of thylakoid membranes was performed as described by Hong and Xu (1999a). The thylakoid pellets were re-suspended in a washing buffer (25 mM Tricine–NaOH, pH 7.8, 10 mM NaF, 1 % bovine albumin, 10 mM NaCl, 5 mM $MgCl_2$). The thylakoid membrane preparation was frozen in liquid nitrogen for further use.

Two-dimensional PAGE: The first-dimension electrophoresis is non-denaturing PAGE. It was carried out at 4 °C on 4-12 % gradient gel and 4 % polyacrylamide stacking gel with the buffer system of Laemmli (1970), but without SDS. A sample of 150 mm^3 thylakoid membrane preparation [12 g(Chl) m^{-3}] was mixed with 30 mm^3 of 10 % DM and 20 mm^3 of 10 % OG. Then the mixture was kept on ice for 20 min. Non-solubilised materials were removed by centrifuging for 2 min at 15 000×*g*. The solubilised thylakoid membrane sample containing 15-20 μg Chl was loaded into each well. The voltage for non-denaturing electrophoresis was 70 V. Running time was 6-7 h.

For second-dimension electrophoresis (SDS/urea/PAGE), the lanes from non-denaturing PAGE mentioned above were excised and loaded onto the stacking gels, then the SDS/urea/PAGE was carried out as described by Hong and Xu (1999a) with a minor modification. A 12 % single concentration gel instead of a linear gradient gel was used.

Western blot: For immunoblotting, proteins resolved by SDS-PAGE were transferred onto a nitrocellulose membrane (*Amersham Pharmacia*, Sweden). The membrane was then assayed with D1-specific antibody (a generous gift from Dr. Jian-Ren Shen) and the antibody binding was detected by an *ECL* system (*Amersham Pharmacia*, Sweden). Densitometric analysis of immunoblots was performed with a *Gel-Doc* system (*Biorad*, USA).

Gel filtration chromatography: BBY-type PS2 membranes were prepared from the saturating irradiance-treated and fully dark-adapted soybean leaves as described by Hong and Xu (1999a). The membranes containing 5 mg Chl were re-suspended with 0.67 cm^3 of 10 % OG and 1 cm^3 buffer solution containing 72 mM MES (pH 6.0), 1.8 M sucrose, 72 mM $MgCl_2$, 18 mM NaCl, and were stirred for 65 min at 4 °C in the dark. Then, the content of the OG-solubilised PS2 membranes was adjusted to 2 mg Chl/cm^3. Equal volume of 2 % DM was added to the OG-solubilised PS2 membranes, and the mixture was stirred for 10 min at 4 °C in the dark. Gel filtration analysis was made by two columns of *Hiload 26/60 Superdex* 200 pg linked serially and equipped to an FPLC set-up according to Shen (1998). The OG- and DM-solubilised PS2 membranes were eluted with a medium containing 30 mM MES (pH 6.0), 150 mM NaCl, and 0.03 % DM at a flow rate of 6.67 mm^3 s^{-1}. The elution was monitored by absorbance at 280 nm.

Results

The proportion of PS2 dimer in total PS2 resolved by non-denaturing PAGE: Peter and Thornber (1991) reported that PS2 dimer and monomer could be separated by Deriphate-PAGE, and the dimers were stable during solubilisation and electrophoresis of thylakoid membranes (Barbato et al. 1995). In our experiments, two forms of PS2 in thylakoid membranes that were solubilised with the detergents DM and OG could also be clearly separated by non-denaturing PAGE without Deriphate. In order to optimise experimental conditions, several concentrations of the detergents were used for solubilisation of thylakoid membranes. A highly repro-

ducible and rather clear electrophoretic pattern (Fig. 1) was observed if thylakoid membranes were solubilised by a detergent mixture containing DM and OG.

There were seven bands in Coomassie brilliant blue-stained native gels for samples from saturating irradiance-treated and dark-adapted leaves (Fig. 1). One of the key components, D1 protein identified by immunoblot with its antibody after two-dimensional PAGE (Fig. 2*A*) existed in bands *4* and *5*. On the basis of molecular mass, PS2 in the band *5* (250 kD) is monomer, and band *4* (425 kD) is possibly PS2 dimer or PS2 monomer with LHC2 attached. If the band *4* is PS2 monomer with LHC2 attached, then band *5* is monomer without LHC2, and all of PS2 is in the form of monomer in soybean leaves. However, the fact that PS2 dimers accounted for about 50 % in total PS2s of BBY particles (Fig. 4, see below) exclude this possibility. So, the PS2 in band *4* is PS2 dimer.

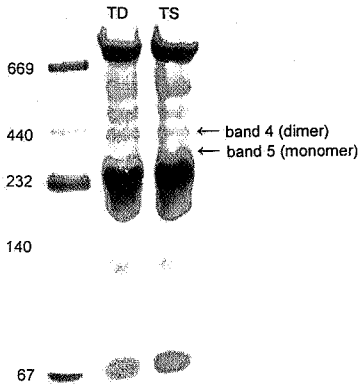

Fig. 1. Non-denaturing PAGE analysis of the Chl-protein complexes in thylakoid membranes from soybean leaves. The thylakoid membrane samples were solubilised by a detergent mixture containing DM and OG. TD – thylakoid membranes from fully dark-adapted soybean leaves; TS – thylakoid membranes from saturating irradiance-treated soybean leaves. Gels were stained by Coomassie brilliant blue dye.

Results of immunoblot analysis after two-dimensional PAGE (Fig. 2*A*) suggested that there was no change in the proportion of PS2 dimer (or monomer) in total PS2 in saturating irradiance-treated leaves as compared with dark-adapted leaves (Fig. 2*B*). In both saturating irradiance-treated and fully dark-adapted leaves, the dimeric form was predominant and the ratio of PS2 dimer to monomer was about 3 (Fig. 2*B*). Also, there was no significant difference in the ratio of PS2 dimer to monomer among those samples treated by the detergents for 1, 10, or 30 min (Fig. 3). These results imply that the presence of the PS2 dimer we observed is not an artifact caused by detergent solubilisation.

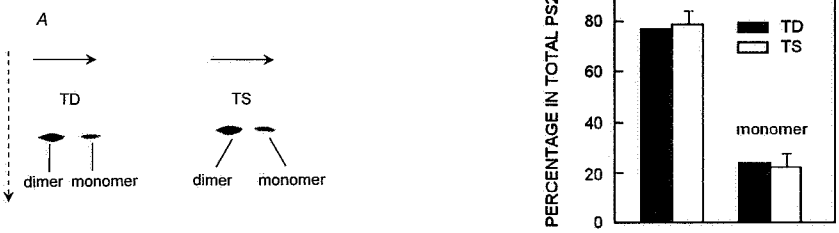

Fig. 2. Immunoblot analysis of thylakoid protein complexes in membrane after two-dimensional PAGE (*A*). The thylakoid membrane samples were fractionated in the first dimension electrophoresis by non-denaturing PAGE, then the lanes were excised and loaded on the second dimension gel (SDS/urea/PAGE) to determine the polypeptide composition of each complex. The amount of PS2 was estimated by immunoblots with D1 protein antibody. The percentage of PS2 dimer/monomer in the total PS2 is shown in *B*. Each column represents the mean of 3 repeats with SE expressed as a bar. The *solid and dash arrows* show the direction from top to bottom in the first and second dimension electrophoresis, respectively.

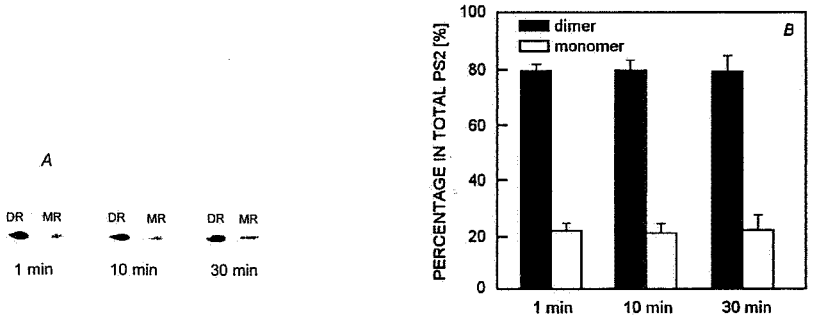

Fig. 3. Effects of the detergent treatment time on proportion of PS2 dimer in total PS2. *A*: the immunoblot analysis of the thylakoid membrane protein complexes after two-dimensional PAGE. *B*: the percentages of the PS2 dimer and monomer. Each column represents the mean of 3 repeats with SE expressed as a bar. DR – dimer, MR – monomer.

The proportion of PS2 dimer in total PS2 resolved by gel filtration chromatography: PS2 dimer and monomer in granal region of thylakoid membranes were isolated by gel filtration chromatography to further explore the effect of saturating irradiance on PS2 oligomeric state. When subjected to size-exclusion chromatography in the presence of DM, BBY particles were resolved into four chlorophyll-enriched fractions (Fig. 4). As shown by Fig. 5, both fractions *1* and *2* consist mostly of PS2 protein components, while the fractions *3* and *4* consist mostly of LHC2. SDS-PAGE analysis and their molecular masses suggest that the fractions *1*, *2*, *3*, and *4* are PS2 dimer, PS2 monomer, LHC2 trimer, and LHC2 monomer, respectively.

The amounts of PS2 proteins in the fractions *1* and *2* were determined according to Bradford (1976). As shown in Fig. 4*C*, PS2 dimers accounted for 53.7 % of total PS2 complex in the samples from saturating irradiance-treated leaves, and 51.1 % in the samples from fully dark-adapted leaves. Therefore, the percentages of PS2 dimer in total PS2 did not change significantly in soybean leaves after saturating irradiation for 3 h.

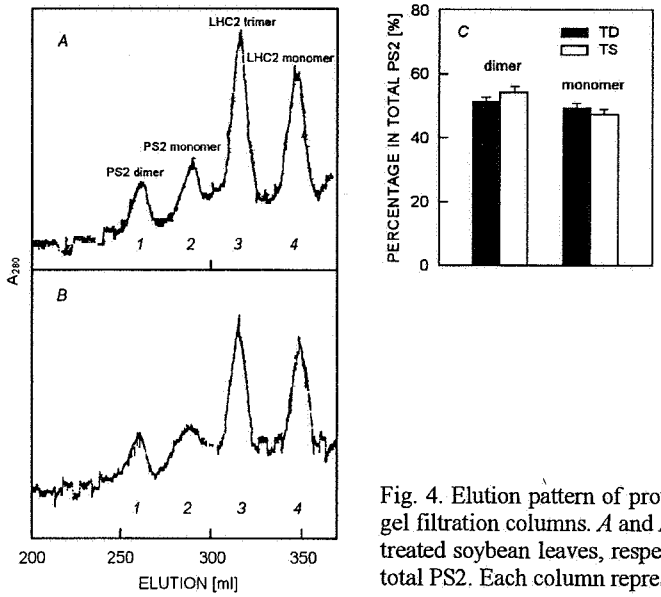

Fig. 4. Elution pattern of protein complexes in OG, DM-solubilised BBY particles from the gel filtration columns. *A* and *B* are samples from fully dark-adapted and saturating irradiance-treated soybean leaves, respectively. *C* shows the percentage of PS2 dimer and monomer in total PS2. Each column represents the mean of 3 repeats with SE expressed as a bar.

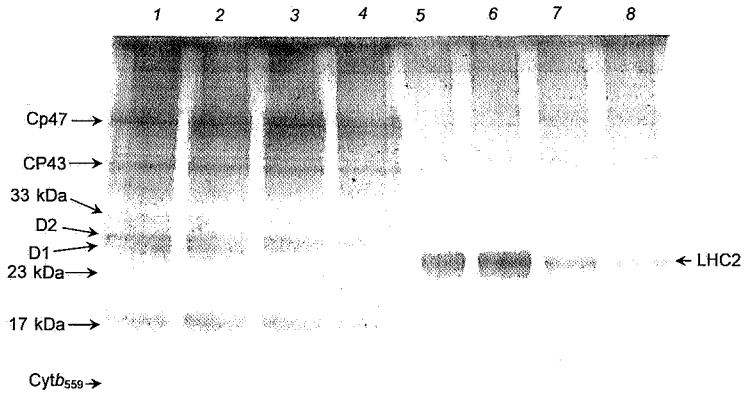

Fig. 5. Silver-stained SDS-PAGE analysis of protein composition of different chlorophyll-rich components eluted from gel filtration columns. Lanes *1*, *3*, *5*, *7*: fractions *1*, *2*, *3*, *4* of the sample from fully dark-adapted soybean leaves; lanes *2*, *4*, *6*, *8*: fractions *1*, *2*, *3*, *4* of the sample from saturating irradiance-treated soybean leaves.

Discussion

Although there is evidence suggesting that PS2 exists mainly as a monomer *in vivo* (Nicholson *et al.* 1996, Shen 1998, Tucker and Sherman 2000), it is accepted that most of PS2s are in the form of dimer in grana and only a small proportion of PS2s exists as monomers in stromal thylakoid membranes (Santini *et al.* 1994, Hankamer

et al. 1997a,b, Rhee *et al.* 1998, Shi *et al.* 2000, Zouni *et al.* 2001). The latter viewpoint is supported by our experimental results obtained by non-denaturing PAGE (Fig. 2) and gel filtration chromatography (Fig. 4). Of course, it has been argued that PS2 dimer may arise through dimerisation of PS2 monomer during detergent solubilisation (Nicholson *et al.* 1996, Shen 1998). If the case is true, the proportion of the PS2 dimer should increase with prolonging the detergent treatment time. In our experiments, however, the ratio of PS2 dimer to monomer did not increase with detergent treatment time increasing within 30 min (Fig. 3). On the contrary, the PS2 dimers may be monomerised during the detergent solubilisation (Hankamer *et al.* 1997b, Bianchetti *et al.* 1998, Zheleva *et al.* 1998). Therefore, our results that most of PS2s were in dimeric form in soybean leaves are not artefact caused by detergent treatment.

In our results the PS2 dimers resolved by gel filtration chromatography accounted for a lower percentage (about 50 %) in total PS2 complexes than that resolved by non-denaturing PAGE (about 75 %). This discrepancy may be explained by the following facts. DM treatment has little effect on oligomeric state of PS2, but OG treatment can lead to monomerisation of PS2 dimer (Santini *et al.* 1994). The ratio of OG to Chl used in solubilisation of BBY particles for gel filtration chromatography was similar to that used in solubilisation of thylakoid membranes for non-denaturing PAGE, but the time of the detergent solubilization of BBY particles (75 min) is much longer than that of thylakoid membranes (20 min). The longer time of solubilisation is most likely to induce monomerisation of some PS2 dimers, thus leading to a lower percentage of the PS2 dimer in BBY particles resolved by gel filtration chromatography. These facts further support the idea that the presence of the PS2 dimer is not a result of dimerisation of PS2 monomer during the detergent treatment, and the PS2 dimer is a main natural form of PS2 complex *in vivo* in soybean leaf.

Some results from *in vitro* system have indicated that photoinhibition involves a process of monomerisation of the PS2 dimer (Barbato *et al.* 1992, Kruse *et al.* 1997). In this process, PS2 dimers dissociate into monomers and these monomers migrate to stroma lamella regions of thylakoid membranes, then degradation and replacement of the D1 proteins occur sequentially. Under anaerobic conditions a fast reversible photoinactivation of PS2 in isolated spinach thylakoids was accompanied by a monomerisation of PS2, and the monomers would re-aggregate into dimers after 3 h recovery in darkness (Mor *et al.* 1997). In our experiment, however, saturating irradiance-caused photoinhibition in soybean leaves did not involve the monomerisation of the PS2 dimer. The conclusion is based on the following facts. First, the ratio of PS2 dimer to monomer in thylakoids from soybean leaves, as measured by the non-denaturing gel electrophoresis, did not decrease after saturating irradiation (Fig. 2). Second, the proportion of the PS2 dimer (or monomer) in total PS2s was unchanged in granal region of thylakoid membranes from saturating irradiance-treated soybean leaves, as shown by the results of gel filtration chromatography (Fig. 4). Third, in the experiments of sucrose density gradient centrifugation, similar results were obtained (data not shown).

It appears that photoinhibition is not necessarily linked with monomerisation of the PS2 dimer. There are two classes of photoinhibition, dynamic and chronic. Chronic photoinhibition is mainly due to photodamage of the photosynthetic apparatus. The dynamic one is principally associated with energy dissipation processes (Osmond 1994). The argument on the change in oligomeric state of PS2 is mainly from chronic photoinhibition. However, the photoinhibition reported here involves neither a conversion of functional PS2 dimer into non-functional PS2 monomer, nor a net loss of D1 protein (Hong and Xu 1999a). The decline in photochemical efficiency in this process may be mainly due to the reversible dissociation of LHC2 from PS2 complex (Hong and Xu 1999b). On the basis of above facts, it is deduced that saturating irradiance-caused photoinhibition is the dynamic one. Therefore, the monomerisation of the PS2 dimer is probably only related to the chronic photoinhibition rather than to the dynamic one.

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