

Stabilization of photosystem two dimers by phosphorylation: Implication for the regulation of the turnover of D1 protein

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Received 21 February 1997; revised version received 7 April 1997

Abstract A general feature of many membrane protein complexes is that they have oligomeric organisation *in vivo*. Photosystem II (PSII) is one such example and the possible functional significance of this is explored in this work. Monomeric and dimeric forms of the core complex of PSII have been isolated from non-phosphorylated and phosphorylated thylakoid membranes prepared from spinach. These complexes had the same complement of proteins including, D1 (PsbA), D2 (PsbD), α -(PsbE) and β -(PsbF) subunits of cytochrome b_{559} , CP47 (PsbB), CP43 (PsbC), 33 kDa (PsbO) extrinsic protein and some other smaller subunits, such as PsbH, but did not contain Cab proteins. D1, D2, CP43 and PsbH were the phosphorylated components. Whether phosphorylated or not, the dimeric form of the PSII complex was more stable than the monomeric form. However, when treated with photoinhibitory light the isolated dimers converted to monomers in their non-phosphorylated state but not when phosphorylated. Phosphorylation, however, did not prevent photoinhibition as judged by the loss of oxygen evolving activity. A model is suggested for the role of PSII phosphorylation in controlling the conversion of dimeric PSII to its monomeric form and in this way regulate the rate of degradation of D1 protein during the photoinhibitory repair cycle.

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Key words: Photosystem II; Phosphorylation; D1 protein; Dimer/monomer interconversion; Turnover; Photoinhibition

1. Introduction

A common feature of a wide range of membrane complexes is that they exist in oligomeric states *in vivo* despite the fact that when isolated as monomers they can carry out their basic enzymic activities. For example, the structure of mammalian cytochrome oxidase recently determined by X-ray crystallography [1] indicates that this complex is dimeric. Dimeric configurations have also been advocated for many other membrane proteins, including cytochrome $b-c$ complexes [2,3], Na^+, K^+ -ATPase [4], ethylene response mediator [5], and intercellular adhesion molecule 1 [6]. In oxygenic photosynthetic organisms it is also widely believed that photosystem II (PSII) normally functions as a dimer [7] while photosystem one in cyanobacteria is trimeric [8]. Almost certainly the oligomerisation of membrane complexes plays a role in controlling and fine tuning their properties. In this paper we discuss the importance of PSII dimer formation in regulating the rapid turn-

over of one of its key components, the D1 protein, and how this regulatory mechanism is further influenced by reversible N-terminal phosphorylation.

PSII is that part of the photosynthetic apparatus that catalyses the unique reactions resulting in the splitting of water and the production of dioxygen and reducing equivalents [9]. It is a membrane located complex made up of more than 20 different subunits. At the heart of the complex is the reaction center composed of the D1 and D2 proteins which are analogous to the L and M subunits of purple photosynthetic bacteria [10]. The reaction center is surrounded by an inner light harvesting antenna system consisting of two chlorophyll α -binding proteins, CP43 and CP47. This core complex, which also contains other non-chlorophyll-binding proteins, is able to catalyse the water oxidation process. In higher plants and green algae, the oxygen evolving core is serviced by an outer antenna system composed of chlorophyll α - and chlorophyll β -binding proteins encoded by a family of nuclear located *cab* genes. The Cab proteins comprise the major light harvesting complex, light harvesting complex II (LHC-II) and the 'linker' proteins, CP24, CP26 and CP29 [11]. Redox controlled reversible phosphorylation occurs in higher plants at the N-terminus of the D1, D2, CP43 and the LHC-II proteins [12]. A non-chlorophyll 10 kDa protein located in the core and encoded by the *psbH* gene also undergoes reversible phosphorylation [12].

Phosphorylation of LHC-II regulates the balance of excitation energy between PSII and photosystem I (PSI) [13] and may also play a role in regulating the efficiency of light harvesting under strong light conditions [14]. The function of the phosphorylation of D1, D2, CP43 and PsbH proteins in higher plant chloroplasts is less clear. Evidence is emerging, however, that the phosphorylation of these components plays a role in the regulation of D1 protein turnover [15–17]. The turnover of this reaction center protein seems to be closely linked to the damage and repair of PSII that occurs as a consequence of its activity as a water oxidase and which increases under conditions of exposure to excess light [18,19]. In two recent studies [20,21] it was demonstrated that phosphorylation of the PSII proteins did not protect against photo-induced damage but significantly reduced the rate of degradation of the D1 and D2 proteins. Moreover, Barbato et al. [22] have suggested that the degradation and turnover of the D1 protein involves a conversion of a dimeric form of the PSII complex into a monomeric form, a process that also involves lateral movement of PSII from the granal to the stromal regions of the chloroplast thylakoid [23].

2. Methods

Thylakoids, PSII enriched membranes and PSII core complexes

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Abbreviations: PSI and PSII, photosystem I and II; LHC-II, light harvesting complex II

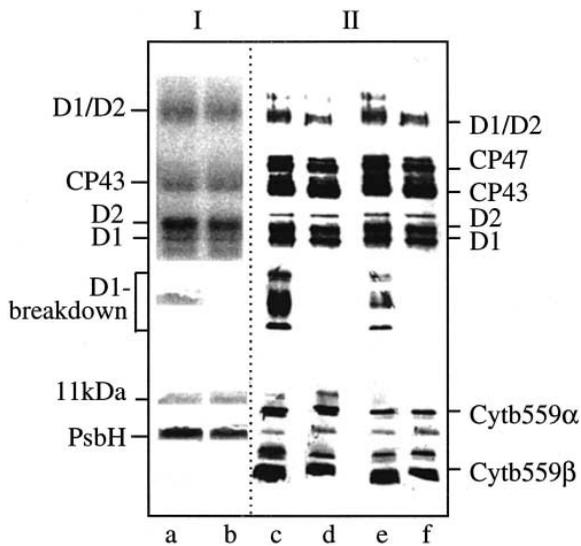


Fig. 1. I: Autoradiogram of samples obtained from ^{32}P Pi phosphorylated thylakoids. (a) PSII monomers, (b) PSII dimers. II: Silver-stained SDS-PAGE of (c) non-phosphorylated PSII monomers, (d) non-phosphorylated PSII dimers, (e) phosphorylated PSII monomers and (f) phosphorylated dimers.

were isolated from spinach (*Spinacea oleracea*) purchased at a local market. PSII enriched membranes (BBYs) were obtained by treating thylakoids with 2% Triton X-100 for 15 min on ice in the dark followed by differential centrifugation according to Berthold et al. [24]. Oxygen evolving dimeric and monomeric PSII cores were isolated from BBYs using *n*-octyl- β -D-glucopyranoside and *n*-dodecyl- β -D-maltoside (DM) together with sucrose density centrifugation according to Hankamer et al. [25]. The structure of these monomeric and dimeric PSII cores have been studied by electron microscopy [26] and in Hankamer et al. [25] their composition and functional activities were reported.

Thylakoids were prepared from spinach leaves and phosphorylated following a modified version of the procedure of Pramanik et al. [27]. Thylakoids were isolated from young spinach leaves after incubating them in the dark for 24 h to promote maximum dephosphorylation. The isolated thylakoids were suspended in a medium containing 0.3 mM PPI and 20 mM NaF and incubated for about 5 min in the dark

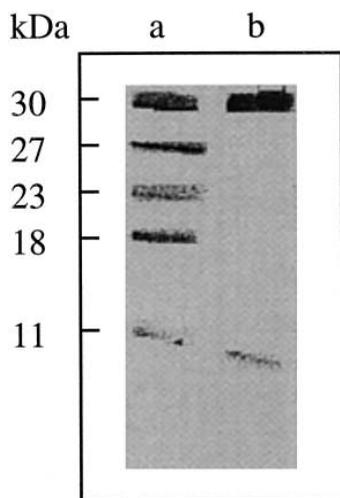


Fig. 2. Immunoblot of (a) PSII monomers and (b) PSII dimers after SDS-PAGE using a mixture of D1 antibody raised against the whole peptide and antibody to the C-terminal of the D1 protein.

and then illuminated for 30 min at room temperature with $300 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$.

Phosphorylation was stopped by centrifugation at 0°C followed by resuspension of the thylakoids into a medium containing 50 mM MES (pH 6.0), 5 mM MgCl_2 , 15 mM NaCl, 0.5 g/l sodium ascorbate and 10 mM NaF.

For radioactive ^{32}P -labelling, ^{32}P Pi (Amersham) was added to the medium to give a concentration of $200 \mu\text{Ci}\cdot\text{mg Chl}^{-1}\cdot\text{ml}^{-1}$. After separation of phosphorylated proteins by SDS-PAGE phosphoimages were recorded using a photoimager (Molecular Dynamics). The same pattern of phosphorylation was observed when $[^{32}\text{P}]\text{ATP}$ was used instead of $[^{32}\text{P}]\text{Ppi}$ except that 5 min incubation was sufficient to obtain the same level of phosphorylation.

SDS-gel electrophoresis was carried out using a 10-17% acrylamide gradient gel containing 6 M urea. Separated proteins were either transferred to nitrocellulose and incubated with monospecific antibodies raised against the C-terminus of the D1 protein (a kind gift from Dr. P. Nixon) and the whole D1 or they were fixed in 40% methanol, 13.5% formalin (37%) for 10 min, reduced with 0.02% dithionite, stained with 0.1% silver nitrate and developed in a medium containing 3% sodium carbonate, 0.05% formalin (37%) and 0.0004% dithionite. The silver-stained gels were analysed by densitometry (Hirschmann Elscript 400).

HPLC was carried out using a size exclusion Zorbax GF-450 column with a flow rate of 1 ml min^{-1} and a monitoring wavelength of 418 nm. The mobile phase contained 0.2 M Tris-HCl (pH 7.2) and 0.05% DM.

3. Results

Using inorganic pyrophosphate (PPI) as the substrate and considering all the known phosphoproteins of spinach thylakoids (LHC-II, CP43, D1, D2 and PsbH) we estimate that

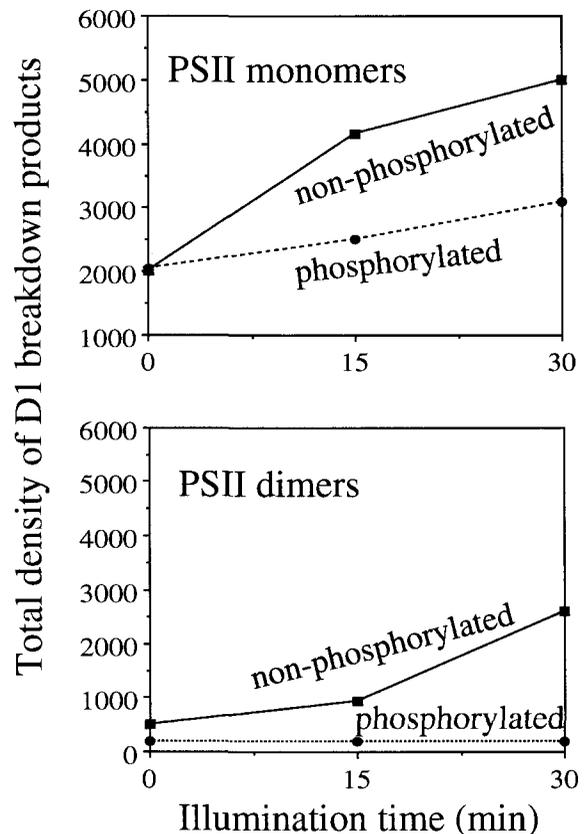


Fig. 3. Accumulation of D1 breakdown fragments obtained by summing the density of immunodetected bands similar to those shown in Fig. 2.

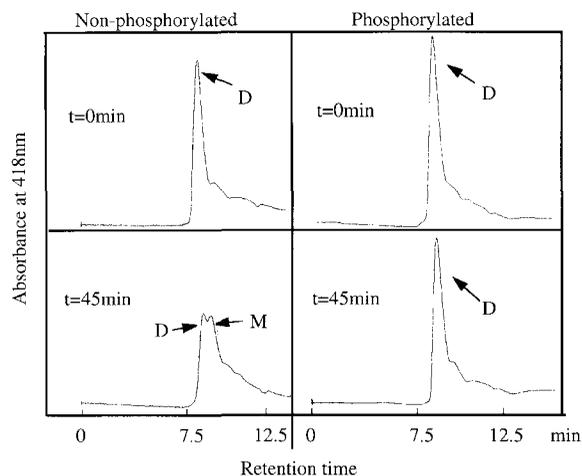


Fig. 4. Size exclusion HPLC elution profiles of non-phosphorylated and phosphorylated dimeric PSII complexes before ($t=0$) and after illumination with photoinhibitory light ($2000 \mu\text{E}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$) for 45 min ($t=45$ min) at 20°C (M = PSII monomer; D = PSII dimer).

80% of the maximum phosphorylation level had been achieved after 60 min of illumination with $300 \mu\text{E m}^{-1}\text{s}^{-1}$ of white light. This slow rate of phosphorylation was also reported previously by Pramanik et al. [28] and is significantly slower than that mediated by ATP.

Non-phosphorylated and phosphorylated thylakoids were solubilised with Triton X-100 and then with OG and DM to produce monomeric and dimeric oxygen evolving PSII core complexes (see Section 2). As can be seen in Fig. 1, in both the monomeric and dimeric forms, the PSII cores contained phosphorylated CP43, D1, D2 and PsbH. Moreover, it was noted that during the isolation procedure the relative amounts of the dimer fraction compared with that of the monomer in the sucrose gradient was considerably higher for phosphorylated compared with the non-phosphorylated states. Also of note and shown in Fig. 1, is that the monomer contained some additional bands in the 18–27 kDa region which were shown by Western blotting to be breakdown products of the D1 protein (Fig. 2). Fig. 2 also shows that no such D1 breakdown fragment was detected in the same molecular mass region for the dimeric form of PSII core except for a very minor phosphorylated band at 11 kDa. This difference in stability between PSII core monomers and dimers was even more pronounced for the non-phosphorylated complexes (Fig. 1).

When the isolated non-phosphorylated monomers and dimers were exposed to high light ($2000 \mu\text{E m}^{-2}\cdot\text{s}^{-1}$) for different periods of time there was an increase in the level of the N- and C-terminal D1 breakdown fragments (Fig. 3). However, as Fig. 3 shows the phosphorylated forms were more resistant to light-induced breakdown. In particular the phosphorylated dimers were the most stable with no D1 breakdown even after 30 min of high light treatment. The increased stability of the isolated PSII core complex in its dimeric and dimeric-phosphorylated forms relative to monomeric forms, was further studied using HPLC size exclusion (see Fig. 4). The HPLC-elution profiles shown in Fig. 4 demonstrate that during 45 min of high light treatment the non-phosphorylated dimer is monomerised. This monomerisation, however, did not occur with phosphorylated dimers. Despite the clear dif-

ference between phosphorylated and non-phosphorylated dimers in terms of their susceptibility to light-induced monomerisation, both were equally sensitive to photoinhibition as judged by the loss of oxygen evolving capacity (see Fig. 5).

4. Discussion

In this paper we confirm that the dimeric oxygen evolving core preparation of PSII isolated using dodecyl maltoside is more stable than its monomeric counterparts [25]. Moreover we present new data which surprisingly show that the PSII core dimer is further stabilised in its phosphorylated state. In this condition it does not readily undergo light-induced monomerisation. In contrast, the non-phosphorylated and phosphorylated forms of the PSII core dimer show identical sensitivity to photoinduced inhibition of oxygen evolution. These findings could have important implications for interpreting the data of others which have suggested that the phosphorylation of core proteins of PSII plays a regulatory role in the repair cycle of photoinhibition that involves the turnover of the D1 protein [20,21]. Previous studies have shown that phosphorylation of PSII proteins does not prevent photoinhibitory damage at the photochemical level but inhibits the subsequent degradation of the D1 protein [19–21]. As a consequence it has been postulated that phosphorylation of PSII functions to prevent disassembly of the complex when the rate of supply of newly synthesised D1 protein is limiting the photoinhibitory repair cycle [19–21]. Moreover the ‘arrested’ phosphorylated form of photochemically damaged PSII could act as a center for quenching excess light energy and thus help protect against further photoinhibitory damage [28–30].

Our results [25,26], and the results of others [7], indicate that PSII exists as a dimer in its normal functional state in the granal regions of higher plant thylakoids. Barbato et al. [22] suggested that the dimeric form dissociated into monomers under photoinhibitory conditions and relocated into the

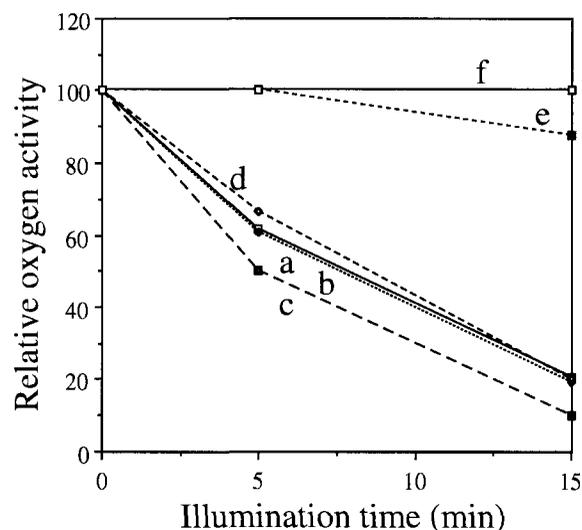


Fig. 5. Effect of photoinhibitory light on oxygen evolution. Phosphorylated and non-phosphorylated samples treated with photoinhibitory light at 20°C for 0, 5 and 15 min. (a) PSII dimers, (b) phosphorylated PSII dimers, (c) phosphorylated PSII monomers, (d) PSII monomers, (e) PSII monomers as a control sample kept at 20°C in the dark and (f) PSII dimers as a control sample kept at 20°C in the dark.

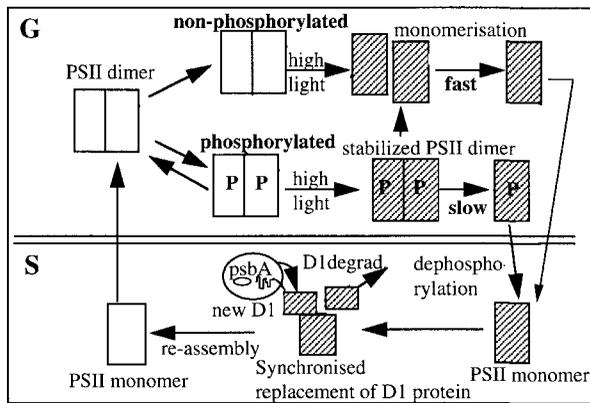


Fig. 6. Scheme for the role of N-terminal phosphorylation of PSII proteins and dimer/monomer interconversion for synchronising the replacement of the D1 protein after photoinhibitory damage. G = Appressed regions of grana, S = non-appressed regions exposed to stroma, P = phosphate group. Shading indicates damaged non-functional PSII or PSII during repair.

unappressed stromal regions were D1 degradation and replacement occurred. It is therefore feasible that monomerisation is necessary to allow reactions involved in D1 degradation and replacement to take place. Our finding that the phosphorylated dimer is resistant to monomerisation suggests that this could be the reason for the phosphorylation-induced inhibition of D1 degradation [20,21]. A model shown in Fig. 6 implies that PSII phosphorylation/dephosphorylation acts *in vivo* to regulate the turnover of the D1 protein. In this model the normal route for D1 protein degradation and replacement involves non-phosphorylated complexes undergoing a dimer to monomer conversion and lateral movement from the granal to the stromal regions of thylakoids, as suggested by Barbato et al. [22]. We propose that this conversion process is inhibited or significantly slowed when the PSII complex is phosphorylated. In the former case it may be necessary to dephosphorylate the inactivated dimer to allow monomerisation to occur. Alternatively, dephosphorylation could take place after the slow monomerisation (see Fig. 6). At present it is not clear whether the conversion from dimer to monomer occurs in the granal, stromal or partition region of the thylakoids. Nevertheless, this change in organization may be a necessary step to facilitate the triggering of proteolytic events which results in degradation of damaged D1 protein and its replacement by newly synthesised protein. If this is the case then the dimer to monomer transition can act as a 'gate' for regulating D1 turnover.

Our model further suggests that the 'gating' mechanism can be controlled by N-terminal phosphorylation of PSII core proteins. This hypothesis is applicable to higher plants where thylakoid membranes are separated into appressed and non-appressed regions and where reversible phosphorylation of PSII polypeptides has been clearly established. However, the model does not apply to cyanobacteria and red algae where the phosphorylation of PSII core proteins has not been shown and where the thylakoid system is not obviously differentiated into two different membrane regions. The model presented also does not take into account the conclusion of Barbato et al. [22] that the monomer to dimer conversion which facilitates D1 protein turnover involves the displacement of CP43 from the core.

The phosphorylation of PSII proteins will add additional negative charge to the surface of the complex. In the case of LHC-II the change in surface charge density due to N-terminal phosphorylation has been suggested to be the destabilising effect which leads to electrostatic repulsion and lateral migration from the granal to the stromal regions [31]. In the case of PSII core proteins the introduction of N-terminal phosphorylation aids stabilisation of the dimer. This effect could also be electrostatic with the additional negative charge increasing short range coulombic attraction with fixed positive charge on the adjacent monomers.

It is worthy of note that the phosphorylation of PSII core protein requires higher light intensities than the phosphorylation of LHC-II. This is reasonable if the function of the former is linked to the regulation of the photoinhibitory repair cycle where the rates of damage and repair become imbalanced at high light intensities. In contrast, LHC-II phosphorylation plays a role in the regulation of energy distribution between PSII and PSI under limiting light conditions.

Acknowledgements: This work was supported by the Biotechnology and Biological Sciences Research Council, The Research Institute of Innovative Technology for the Earth and the European Human Capital and Mobility Program (ERB 4050 PL94-0237) (O.K.).

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