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Subsequent events to GTP binding by the plant PsbO protein: Structural changes, GTP hydrolysis and dissociation from the photosystem II complex

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Abstract

Besides an essential role in optimizing water oxidation in photosystem II (PSII), it has been reported that the spinach PsbO protein binds GTP [C. Spetea, T. Hundal, B. Lundin, M. Heddad, I. Adamska, B. Andersson, Proc. Natl. Acad. Sci. U.S.A. 101 (2004) 1409–1414]. Here we predict four GTP-binding domains in the structure of spinach PsbO, all localized in the β-barrel domain of the protein, as judged from comparison with the 3D-structure of the cyanobacterial counterpart. These domains are not conserved in the sequences of the cyanobacterial or green algae PsbO proteins. MgGTP induces specific changes in the structure of the PsbO protein in solution, as detected by circular dichroism and intrinsic fluorescence spectroscopy. Spinach PsbO has a low intrinsic GTPase activity, which is enhanced fifteen-fold when the protein is associated with the PSII complex in its dimeric form. GTP stimulates the dissociation of PsbO from PSII under light conditions known to also release Mn^{2+} and Ca^{2+} ions from the oxygen-evolving complex and to induce degradation of the PSII reaction centre D1 protein. We propose the occurrence in higher plants of a PsbO-mediated GTPase activity associated with PSII, which has consequences for the function of the oxygen-evolving complex and D1 protein turnover.

Keywords: Photosystem II; PsbO protein; GTPase; Oxygen-evolving complex; D1 protein

1. Introduction

Photosystem II (PSII), a multi-subunit membrane–protein complex, uses light energy to perform the most thermodynamically demanding and unique reaction of photosynthesis, namely the oxidation of water to molecular oxygen and reducing equivalents. The crystal structure of PSII complex isolated from the cyanobacterium *Thermosynechococcus elongatus* at 3.5-Å resolution provided the first complete model of the oxygen-evolving complex (OEC), composed of a cluster of four manganese and one calcium ions, and the associated protein environment [1]. Most recently, a more refined structure of the PSII complex from the same organism, became available [2]. In plants and green algae, the OEC includes three extrinsic proteins, PsbO, PsbP and PsbQ (see

Abbreviations: CD, circular dichroism; Chl, chlorophyll; DCBQ, 2,6-dichloro-*p*-benzoquinone; G domain, guanine nucleotide-binding domain; GAP, GTPase-activating protein; GDI, guanine nucleotide dissociation inhibitor; GEF, guanine nucleotide exchange factor; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; LHC, light-harvesting complex; MES, 4-(*N*-morpholino) ethanesulfonic acid; OEC, oxygen-evolving complex; PSII, photosystem II; SDS, sodium dodecyl sulphate; TLC, thin-layer chromatography; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; Trp, tryptophan

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[3,4] for reviews). Cyanobacteria have PsbU and PsbV, as well as PsbO as OEC extrinsic proteins, although there is evidence that they may also have homologs of PsbP and PsbQ associated with their PSII complexes [5]. The PsbO protein is conserved in all known oxygenic photosynthetic organisms, showing a 40–50% sequence homology between cyanobacteria and higher plants [6].

A great deal of structural information about the PsbO protein isolated from plants has been gathered in the last decades from various biophysical approaches [7–10]. 'Natively unfolded' versus 'molten globule' states of the PsbO protein have been compared and discussed in terms of flexibility and interaction with other proteins [11]. De Las Rivas and Barber [6] have conducted a detailed analysis of the *in situ* PsbO structure based on the X-ray work on the cyanobacterial PSII [1]. These analyses strongly indicate that the PsbO protein of plants and green algae have a very similar structure to that of cyanobacterial PsbO, namely a β-barrel composed of eight anti-parallel β-strands (β1–β8), three α-helices (h1–h3), hydrophilic loops and turns. An extensive loop joining β-strands 5 and 6 (β5–β6) forms a 'head' domain to the β-barrel, and plays important roles in binding of PsbO to the lumenal surface of the PSII complex as well as in stabilizing the Mn_4Ca-cluster of the OEC. The N- and C-termini are close to each other, and located towards the lumenal end of the β-barrel domain. A loop joining β-strands 1 and 2 (β1–β2) is proposed to stabilize the dimeric structure of the PSII complex [6].

PsbO is the minimum and the most crucial PSII lumenal extrinsic component for an adequate function of the water oxidation in PSII (see [3] for a review). Most recently, it was shown to fine tune the pKa in the proton exit pathway [1] from the Mn_4Ca-cluster to the lumenal surface [12]. Spectroscopy studies have indicated that the isolated plant PsbO protein undergoes calcium-induced structural changes, which may be important in regulating its interaction with PSII [13,14]. The same conclusion was also drawn by Shutova et al. [15], who found that Ca^{2+} and especially Mn^{2+} binding to PsbO affected association/dissociation with the PSII complex and OEC activity. A calcium-binding site has been recently identified in the cyanobacterial PsbO protein, located in the upper part of the β-barrel domain close to the lumenal exit of a putative proton channel [16]. It was also reported that GTP is produced inside the plant thylakoid lumen, where it binds with high affinity to the PsbO protein associated with the PSII complex [17]. This latter observation may have significance for light-mediated signal transduction events across the thylakoid membrane, including the GTP-dependent degradation of the PSII reaction centre D1 protein under high [18] and low [19] light conditions.

There is a wealth of information available about signal-transducing GTPases in plants [20,21], which can be compared with the observations for the PsbO protein. The small GTPases are usually of 18–33 kDa, can be found both in soluble and membrane-associated forms, and share several guanine nucleotide-binding (G) domains [22,23]. Their GTP-hydrolyzing activity requires Mg^{2+} ions, and is regulated via interaction with auxiliary components, namely a GTPase-activating protein (GAP), a guanine nucleotide exchange factor (GEF), and a guanine nucleotide dissociation inhibitor (GDI) [20,24].

Here we present experimental evidence for GTP binding and hydrolysis by the spinach PsbO protein. We show that, together with light, GTP hydrolysis regulates the attachment of PsbO to PSII, and may play a role in PSII function and repair. Furthermore, based on the partial homology of putative G domains in the plant PsbO sequences to those of small GTPases, we model the GTP-binding site in the homologous 3D-structure of the spinach protein.

2. Materials and methods

2.1. Plant material, isolated PSII preparations and purified PsbO protein

Spinach (*Spinacea oleracea*) plants were grown hydroponically as previously described [17]. PSII membranes were prepared according to [25], and stored in 50 mM MES–NaOH (pH 6.5), 400 mM sucrose, 10 mM NaCl, and 5 mM $MgCl_2$ (buffer A). NaCl- and $CaCl_2$-washed PSII membranes were prepared by incubation of PSII membranes (0.5 mg of Chl ml^{-1}) with buffer A containing 1.5 M NaCl or 1.5 M $CaCl_2$ for 30 min in darkness followed by centrifugation (40,000×*g*, 30 min). The membranes were once washed and resuspended in buffer A. PSII core complexes were isolated from PSII membranes by perfusion chromatography [26], and finally resuspended in 50 mM MES–NaOH (pH 6.0), 2.0 M glycine betaine, 10 mM NaCl, 5 mM $MgCl_2$, 5 mM $CaCl_2$, and 0.01% *β*-dodecyl maltoside (buffer B). PSII complexes containing the light-harvesting subunits (LHCII–PSII) were isolated from thylakoid membranes by detergent solubilization followed by sucrose density gradient centrifugation [27], and stored in buffer B. Chlorophyll (Chl) concentration was measured according to [28].

Purified PsbO protein was prepared as described [15] by two consecutive treatments of PSII membranes with buffer A containing 1.5 M NaCl and 20 mM Tris–HCl (pH 9.0) containing 1 M KCl, each step for 1 h in darkness on ice followed by centrifugation (40,000×*g*, 30 min). The final supernatant was concentrated, desalted and the buffer exchanged for 50 mM HEPES–NaOH (pH 7.4) and 5 mM $MgCl_2$ by centrifugation in an Amicon ultracentrifugal filter device (10 MWCO membranes). Prior to circular dichroism (CD) measurements, the purified PsbO protein was dialyzed against 2 mM HEPES–NaOH (pH 7.4) and concentrated. To prepare recombinant protein, the spinach *psbO* gene was cloned in the pET-32b(+) vector (Novagen), and the protein was purified according to pET system manual. The protein concentration was measured spectrophotometrically at 276 nm by using an extinction coefficient of 16 mM^{-1} cm^{-1} [29], and in addition by the standard method [30].

2.2. Circular dichroism and fluorescence measurements

UV circular dichroism (CD) spectra were monitored with a Jasco J-800 spectropolarimeter at the following optical path lengths: 1 mm in the region of 240–198 nm and 0.2 mm in the region of 198–184 nm. Calibration was carried out using a freshly prepared solution of d-camphor-10-sulfonate. For the far-UV (184–220 nm) CD measurements, the purified PsbO protein was diluted with 2 mM HEPES–NaOH (pH 7.4) and 1.5 mM $MgCl_2$ (buffer C) to a final concentration of 0.2 mg ml^{-1} (7.2 μM) in a volume of 250 μl. Two scans (0.2 mm) were made at +25 °C for each nucleotide added (150 μM GTP, GDP or GTPγS). Experimental data were smoothed by the fast Fourier transformation filter function (OriginPro 7.5, PriginLab Corporation, Northampton). Room temperature absorption and fluorescence spectra were measured using a Shimadzu MPS-2000 spectrophotometer and a Fluoro Max-2 spectrofluorimeter, respectively. The intrinsic protein fluorescence was excited at 276 nm, and the spectra were recorded between 290 and 400 nm in a 1.1 ml mixture of PsbO protein (0.066 mg ml^{-1}, 2 μM) in buffer C and 15 μM GTP.

2.3. GTP hydrolysis assay

Purified or recombinant PsbO protein (0.14 mg ml^{-1}) was incubated with 0.2 μM [α-^{32}P]GTP (Amersham, 3.5 Ci/mmol, 1 Ci=3.7×10^{10} Bq) for the indicated periods of time at +25 °C. The enzymatic reaction was stopped by the addition of an equal volume of 4 N HCOOH. Three microliters (75 nCi) of the resulting mixture was applied to a poly(ethyleneimine)-cellulose plate (Merck), and the reaction products were separated by thin-layer chromatography (TLC) with 0.75 M KH_2PO_4 (pH 3.65) as elution buffer. The radioactive GTP and GDP spots were detected using a Phosphorimager model FLA-5100 (Fuji) and quantified using ImageReader and MultiGauge softwares (Fuji). GTP hydrolysis was measured under similar conditions in various PSII preparations (0.2 mg of Chl ml^{-1}) incubated in darkness in 20 mM MES–NaOH (pH 6.0) or 20 mM HEPES–NaOH (pH 7.4), 100 mM sucrose and 5 mM $MgCl_2$.

2.4. Dark versus light-induced release of the PsbO protein

NaCl-washed PSII membranes (lacking the PsbP and PsbQ proteins) were incubated in an ELISA plate (150 μl volume) at 0.075 mg of Chl ml^{-1} for 30 min in a water bath at +22 °C in darkness or under moderate light (120 μmol photons m^{-2} s^{-1}) from a KL2500 lamp (Schott). This incubation was conducted in 20 mM MES–NaOH (pH 6.0) or 20 mM HEPES–NaOH (pH 7.4), 100 mM sucrose, 5 mM $MgCl_2$, 0.1 mM 2,6-dichloro-*p*-benzoquinone (DCBQ), and 0.5 mM ferricyanide in the absence or presence of 200 μM GTP. The samples were centrifuged, and the pellets and supernatants analyzed by Western blotting with anti-PsbO antibody.

For studies of PsbO protein release induced under photoinhibitory conditions, LHCII–PSII supercomplexes (0.2 mg of Chl ml^{-1} in buffer B containing 1.0 M glycine betaine) were kept in darkness or illuminated (100, 200 or 1000 μmol photons m^{-2} s^{-1}) for 30 min in an ELISA plate (50 μl final volume) in a water bath at +22 °C. Where indicated, 200 μM GTP were added at the onset of illumination. Oxygen evolution activity was measured with a Clark-type electrode (Hansatech, Kings Lynn, England) under saturating visible light at +22 °C using 5 μg of Chl ml^{-1} in buffer B and 0.5 mM phenyl-*p*-benzoquinone as electron acceptor. For Western blot analyses, the samples were diluted with 150 μl buffer B containing 1.0 M glycine betaine, and concentrated by ultrafiltration in an Amicon ultracentrifugal filter device (100 MWCO membranes). The filtrates were mixed with 0.8 ml 100% (vol/vol) acetone and incubated at −20 °C for 20 min before centrifugation and solubilization of the pellet in SDS-sample buffer. The filtrates and corresponding concentrates were analyzed by Western blotting with anti-PsbO and D1 antibodies.

2.5. Protein analysis

SDS/urea/PAGE and Western blotting were performed as previously described [18]. The purity of the purified PsbO protein was verified by silver staining of SDS-gels and also by photoaffinity labeling with radioactive GTP [17]. Where indicated, antibodies raised against the spinach PsbO and D1 proteins were used. An antibody raised against a synthetic peptide of the β-subunit of the ATP-synthase was obtained from AgriSera (Umeå, Sweden).

3. Results

3.1. Prediction of G domains in the structure of the PsbO protein

It has been shown previously that the PsbO protein of spinach binds GTP with high affinity [17]. We have therefore searched for potential G domains in the amino acid sequence of PsbO from this higher plant as well as from the green alga *Chlamydomonas reinhardtii* and cyanobacterium *Thermosynechococcus elongatus*. Many GTP-binding proteins have a phosphate-binding loop (P-loop or G1), consisting of a glycine-rich sequence followed by a conserved lysine and a serine or threonine (GX_4GKS/T, PROSITE PS00017). The lysine residue of the G1 domain may interact directly with one of the phosphate groups of the bound GTP [31,32]. Furthermore, the same residue interacts with a negatively charged residue from the GEF [33]. The G1 domain was not found 100% conserved in the three analyzed PsbO sequences. The spinach PsbO has a 50% similar motif in the N-terminal part between residues 42 and 48 (Fig. 1A): pGKYTaKk (the lower case letter indicates a non-conserved residue), and contains the essential lysine residue. The spacing between glycine G43 and the downstream alanine A47 by only three residues and the replacement of the conserved serine/threonine in the GKS/T signature with lysine, implies presence of a non-canonical form of the P-loop, as in some families of P-loop NTPases [23]. Notably, the GK pair (residues 43–44) is also present in the P-loop of septin GTPases [23], such as the well-studied Toc34 GTPase of the chloroplast protein translocon [34]. It was suggested that the proteins containing this GK pair may function as dimers [23]. In the 3D homology model of the spinach PsbO based on the crystal structure of the cyanobacterial protein [13], the putative G1 domain corresponds to a short loop between helix h2 and strand β1 (Fig. 1A and B, right panel), similarly to the case of canonical GTPases [22].

The three dimensional shape of the P-loop is identical in the GDP- and GTP-bound forms of GTPases [22]. By contrast, two other well-conserved domains involved in binding of the γ-phosphate of GTP and the catalytic Mg^{2+} ion, often named G2 (a conserved threonine residue) and G3 (DX_2G), respectively, confer different conformations of residues in the flanking regions (Switches I and II) in the GDP- and GTP-bound states of the protein [22,23]. The threonine T84 residue in spinach located in the putative strand β2 (Fig. 1A and B, right panel) fulfils the requirements of a G2 domain. The region rich in aspartate, glutamate and glycine residues, also called plant E-rich region [6], contains the signature for the G3 domain (Fig. 1A, D86–G90 in spinach). Notably, the same region has been previously proposed to be involved in calcium binding [13]. In the structural homology model, the Switches I and II correspond to the regions flanking G2 and G3 domains, namely the long β1–β2 and the short β2–β3 loops (Fig. 1B, left panel). No specific interactions are documented for the latter loop. The long and flexible β1–β2 loop is involved in interactions at the lumenal surface with CP47 of the other PSII monomer which constitutes the dimeric complex [6]. Thus, drastic changes in conformation of the Switches I and II upon GTP hydrolysis may decrease the area of interconnection between the two PSII monomers at the lumenal surface.

A fourth conserved domain (G4), involved in the specific binding of the guanine ring, usually consists of hydrophobic and polar amino acids followed by (N/T)KX(D/E) motif [22,23]. The G4 domain is found in the β4–β5 loop of the spinach PsbO sequence between residues 136–139 (Fig. 1A and B, right panel): gKPE (the lower case letter indicates a non-conserved residue). Notably, the asparagine residue is not conserved in PsbO, as in the case of the Toc34 GTPase [22,34], implying unusual nucleotide binding properties [17,35].

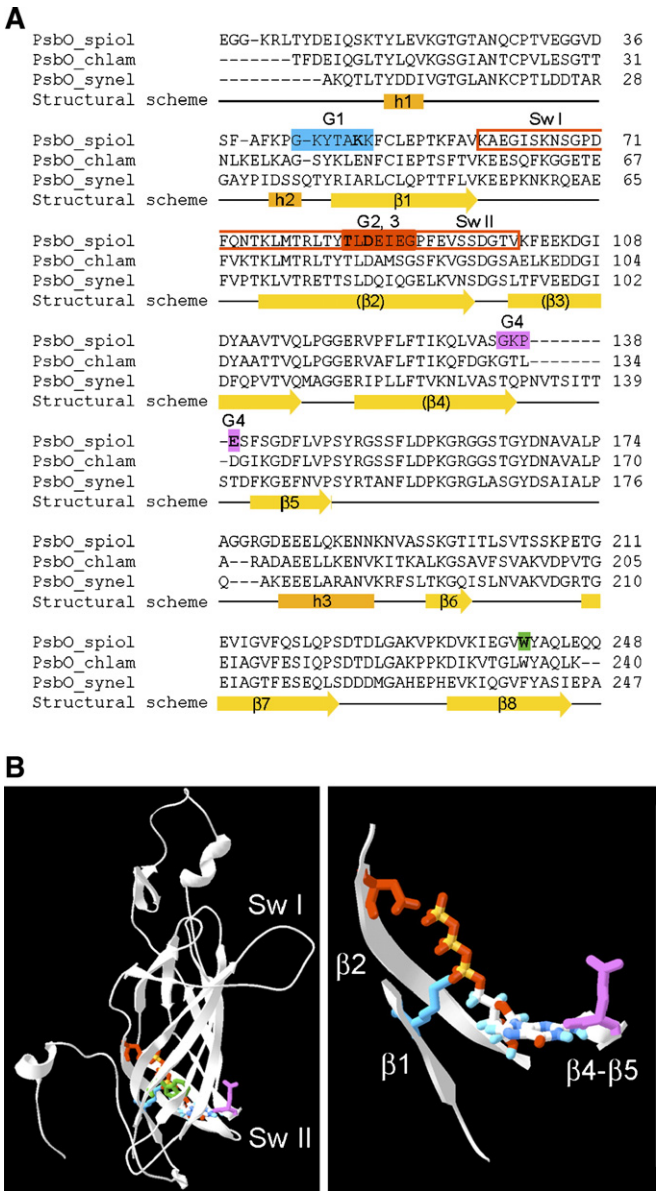

Fig. 1. Prediction and location of GTP-binding domains in the structure of the PsbO protein. (A) The following PsbO amino acid sequences were aligned using ClustalW software (http://www.ebi.ac.uk/clustalw): from *Spinacia oleracea* (spiol), from *Chlamydomonas reinhardtii* (chlam) and from *Thermosynechococcus elongatus* (synel). The P-loop (G1) was predicted using PROSCAN software (http://npsa-pbil.ibcp.fr/NPSA/npsa_proscan.html). The predicted locations of conserved motifs for GTP binding [22] are indicated as highlighted amino acid residues in blue (G1), red (G2 and G3) and magenta (G4). The critical residues in each region are marked in bold. The tryptophan residue in the C-terminus is given in green. The secondary structure elements for the cyanobacterial PsbO [6] are shown below the sequences. (B) Left panel: location of G domains in the structural homology model of the spinach PsbO protein, based on the X-ray structure of cyanobacterial PsbO [1] and built using the Swiss-Model program [49]. Right panel: only the region containing the predicted G domains is shown. Similar colours as in (A) were used to highlight the critical residues of the G domains. Using Swiss-Pdb Viewer 3.1 software, a GTP molecule (ball-and-stick model) is fitted at distances 1–2 Å from the critical residue of each G domain. Switches (Sw) I and II may have different conformations in the GTP- and GDP-form of the protein.

Fig. 1B (left panel) shows the proposed orientation of a GTP molecule in the structural homology model of the spinach PsbO protein, namely inside the β-barrel domain close to the lumenal end of the protein. None of the putative G domains is conserved in the corresponding regions of the *Chlamydomonas* or *Thermosynechococcus* PsbO proteins (Fig. 1A). On the other hand, all four G domains predicted in spinach are conserved in other plant species such as Arabidopsis, tobacco, tomato and potato (data not shown), suggesting that GTP binding to the PsbO protein may be a characteristic feature of higher plants only.

3.2. GTP-induced changes in the PsbO structure in solution

In order to study the interaction of GTP with PsbO, secondary structural changes were investigated by far-UV CD spectroscopy upon the addition of the nucleotide to the spinach protein in solution. The spectra presented in Fig. 2A reproduced the typical characteristics of a properly folded PsbO, containing a maximum at 195 nm and a minimum at 208 nm [7,9]. Addition of GTP changed the spectrum 1, recorded in the presence of Mg^{2+}, such that both maximum and minimum showed less pronounced ellipticity (spectrum 2). Addition of GTP in the absence of Mg^{2+} did not induce any CD changes in spectrum 1 (data not shown), consistent with the requirement of Mg^{2+} ions for the high affinity binding of GTP by the small GTPases [22]. The CD spectra in the presence of MgGDP or the slowly hydrolyzable MgGTPγS were also similar to the control spectrum 1 (data not shown). Thus, only MgGTP induced significant changes in the secondary structure of the PsbO protein in solution, possibly in the predicted Switches I and II (Fig. 1B, left panel), which have different conformations in the GDP- and GTP-bound states.

Intrinsic fluorescence of the PsbO protein in solution is assigned to the only tryptophan residue conserved in all eukaryotic PsbO (Fig. 1A, W241 in spinach), with a maximum at 329.5 nm, and to tyrosine(s) with a maximum at 306 nm ([36] and Fig. 2B spectra 1 and 2). As shown by the difference spectrum −/+GTP (Fig. 2B spectrum 3), only the tryptophan emission maximum was affected and slightly shifted to shorter wavelengths when MgGTP was added, indicating that W241 becomes more buried in the hydrophobic core of the protein in the presence of MgGTP. The location of W241 in close vicinity to both G1 and G2–G3 domains could explain the observed changes in the tryptophan fluorescence.

Only around 5% of the total number of amino acid residues of PsbO may be directly involved in coordinating various parts of the GTP molecule (Fig. 1). Although not dramatic, the changes observed in the CD and tryptophan fluorescence spectra are specific for MgGTP, and are attributed mostly to modifications in the tertiary structure of protein domains containing or located close to the predicted G domains.

3.3. GTPase activity of the PsbO protein in purified and PSII-associated form

To verify that PsbO cannot only bind but also hydrolyze GTP, we incubated the protein purified from spinach PSII

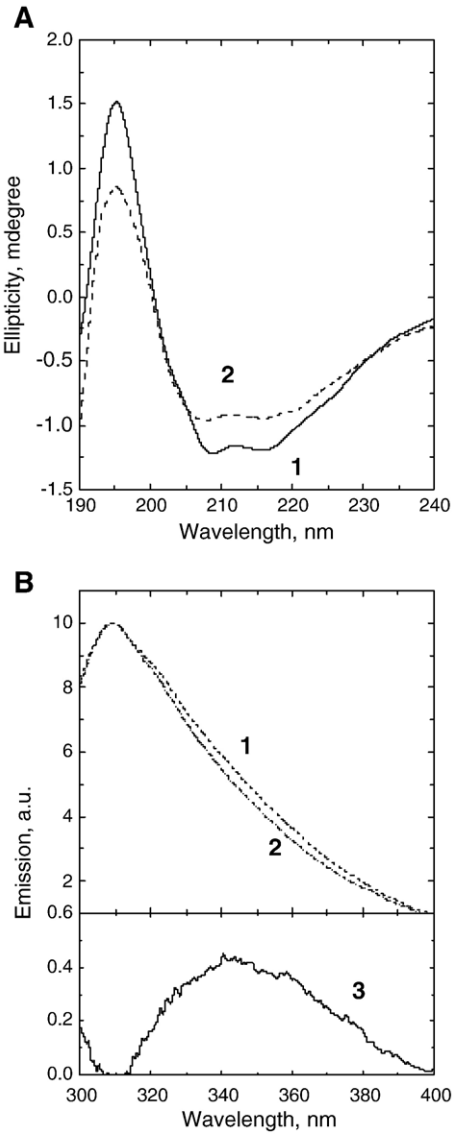

Fig. 2. GTP-induced changes in the structure of the PsbO protein in solution. (A) Far-UV CD spectra of the PsbO protein (7.2 μM in buffer C) in the absence (1) and presence (2) of 150 μM GTP. (B) Normalized room temperature intrinsic fluorescence of the PsbO protein (2 μM) in the absence (1) and presence (2) of 15 μM GTP. Excitation wavelength was 276 nm. Insert (3) represents the difference fluorescence spectrum (−/+GTP). Each of the shown CD and fluorescence spectra represents average of five independent experiments.

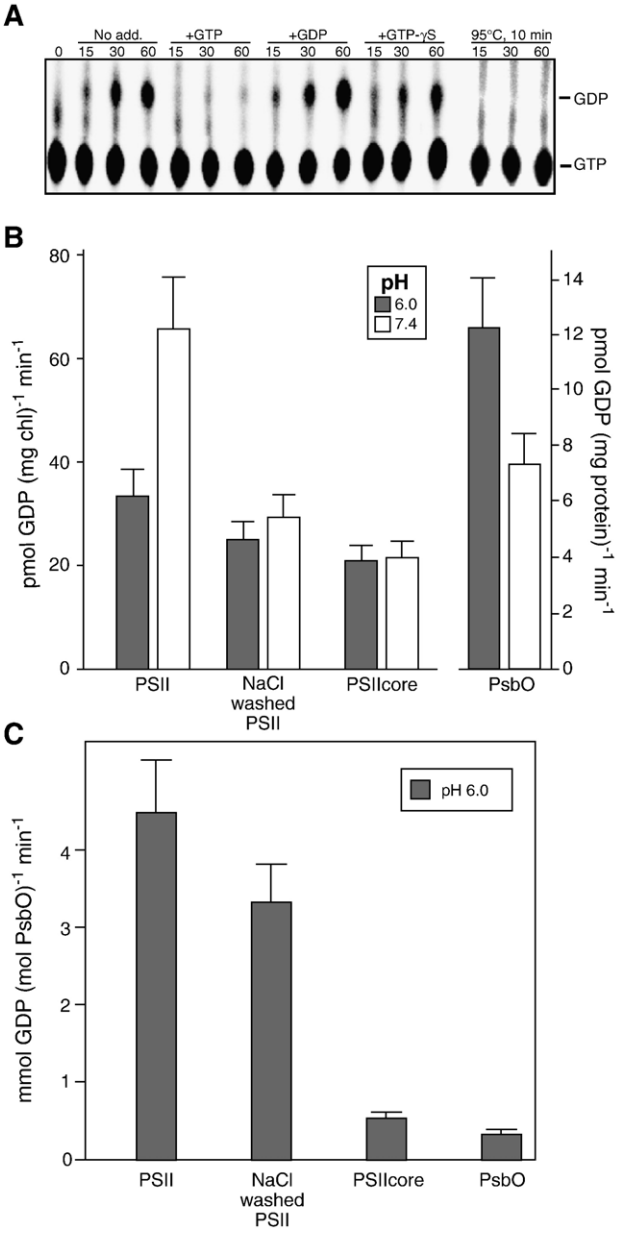

Fig. 3. GTP hydrolysis by isolated and PSII-associated PsbO protein. (A) 1.4 μg PsbO protein was incubated with 0.2 μM ([α-^{32}P]GTP (3.5 Ci/mmol) in 10 μl buffer C for the indicated periods of time at +25 °C, in the absence (No add.) or presence of 50 μM non-labeled nucleotides (GTP, GDP, GTPγS). Control sample was PsbO protein boiled for 10 at +95 °C. The enzymatic reaction was stopped by the addition of an equal volume of 4 N HCOOH. The guanine nucleotides were separated by TLC, and the radioactive species were detected by phosphorimaging. (B) GTP hydrolysis was assayed as in (A), in isolated PSII membrane preparations (0.2 mg of Chl ml^{-1}) untreated or washed with 1.5 M NaCl or 1.5 M $CaCl_2$, in PSII cores (0.2 mg of Chl ml^{-1}), and in purified PsbO protein (0.14 mg protein ml^{-1}) incubated in MES (pH 6.0) or HEPES (pH 7.4) buffers. The GTPase activity was expressed as amount of GDP produced per mg Chl (left panel) or per mg protein (right panel). (C) The GTPase activities determined at pH 6.0 (as in B) were expressed per mol PsbO. For this purpose, comparative immunostaining using known amounts of PsbO was used to calculate the amount of PsbO per mg Chl in each type of PSII preparation. The standard deviations were calculated for data obtained from three independent preparations.

membranes with [α-^{32}P]GTP for increasing periods of time. The nucleotides were separated by TLC, and the radioactive species were detected by phosphorimaging. As shown in Fig. 3A (No add.), GDP was detected after 15 min of incubation, and its amount increased up to 60 min. To test the specificity of the PsbO protein for GTP hydrolysis, we performed experiments in the presence of excess of non-labeled guanine nucleotides (Fig. 3A, +GTP, +GDP and +GTPγS). Only GTP competed efficiently with the production of GDP whereas GDP did not affect at all the hydrolyzing activity. The slowly hydrolyzable GTPγS reduced by about 40% the GDP levels detected in the control (No add.). No residual GTPase activity was detected in the heat-denatured (+95 °C for 10 min) PsbO (Fig. 3A),

indicating that the above measured GDP production was the result of an enzymatic activity dependent on the conformation of the protein. The calculated specific activity at pH 6.0 was 12 pmol GDP (mg protein)$^{-1}$ min^{-1} (Fig. 3B, right panel) or 0.33 mmol GDP (mol PsbO)$^{-1}$ min^{-1} (Fig. 3C). The GTPase activity of bacterial-expressed recombinant spinach PsbO was similar to that measured for the PsbO protein purified from PSII membranes (data not shown). This excludes the possibility of GTP hydrolysis being due to contaminants, and implies that the two types of PsbO are structurally and functionally the same, in line with previous reports ([37] and references therein). Thus, our data strongly support the notion that spinach PsbO has a GTPase activity.

GTP hydrolysis by the PsbO protein was also assayed using several types of PSII preparations isolated from spinach, such as PSII membranes, before and after washing with NaCl or $CaCl_2$, and isolated PSII core complexes (Fig. 3B, left panel). There are major differences between these types of preparations in both oligomerization state and biochemical composition. First, the PSII membranes are fragments from the grana regions of the thylakoid membrane enriched in PSII [25]. In this membrane environment, PSII exists as dimeric complexes being the natural state *in vivo*, and containing all the OEC extrinsic proteins. Second, the NaCl- and $CaCl_2$-washed PSII membranes are both depleted of PsbP and PsbQ, but retain or lack the PsbO protein, respectively [3]. Finally, the isolated PSII core complexes used here were monomeric, and largely depleted of the PsbP and PsbQ proteins as well as of the LHCII subunits [26]. The GTPase activities of various PSII preparations (expressed per mg Chl) and of isolated PsbO (expressed per mg protein) were measured during incubation in darkness at pH 6.0 and 7.4 (Fig. 3B). These values were reported as the physiological pH under *in vivo* light and dark conditions, respectively [38]. Among the tested preparations, only PSII membranes and purified PsbO protein showed a remarkable difference in GTP hydrolysis at the two analyzed pH values. PSII membranes were more active at pH 7.4 whereas purified PsbO had higher activity at pH 6.0. The PsbO-related activity of PSII membranes at pH 6.0 was retained in NaCl-washed PSII membranes (Fig. 3B, left panel). No significant GDP production was detected in $CaCl_2$-washed membranes (data not shown), consistent with the absence of the PsbO protein in this type of preparation. Neither the NaCl-treated PSII membranes nor PSII cores showed a pH dependence of the GTPase activity (Fig. 3B, left panel). The reason for the different responses to pH of various preparations could be, as recently suggested [15], that the local pH changes are more important than the bulk pH for the GTPase activity of the bound PsbO form.

For a better comparison between the GTPase activities of PsbO in isolated and bound forms, we determined the amount of PsbO per mg Chl loaded in the various assayed PSII preparations, by using Western blotting and a titration curve with purified PsbO protein. Assuming that spinach PsbO has a molecular mass of 26.7 kDa, the activities at pH 6.0 expressed per mg Chl (Fig. 3B, left panel) were recalculated and expressed per mol PsbO (Fig. 3C). In this case, the PsbO subunit of a monomeric PSII (core) complex had a poor GTPase activity, i.e. seven to nine-fold lower as compared to the activity in the PSII dimeric form (PSII membranes), and only two-fold higher than that of purified PsbO protein. The possibility of involvement of other extrinsic components such as PsbP and PsbQ in regulating the GTPase activity of PsbO is not likely since the values obtained in NaCl-washed PSII membranes were only 1.4-fold lower than in untreated membranes at pH 6.0 (Fig. 3C). Western blot analyses of purified PsbO protein as well as of the PSII cores and membranes with an antibody against the β-subunit of the ATP-synthase excluded the possible contamination with thylakoid bound ATPase as a source of the assayed GTP hydrolysis (data not shown). Taken together, the results presented in Fig. 3 show that the PsbO protein has a low intrinsic GTPase activity, which is considerably enhanced when associated to a PSII dimer. We suggest that an optimal function of PsbO as a GTPase requires a certain conformation of the PsbO protein, which is readily achieved upon binding to the PSII complex [11,37,39]. PsbO association with PSII, the dimeric organization of PSII, and other yet unknown (lumenal) factors may act as regulatory components of the GTP-hydrolyzing activity.

3.4. GTP stimulates the light-induced release of the PsbO protein

The sequence of events in the activation–inactivation cycle of small GTPases is well documented [20,21]. Following GTP hydrolysis, the inactive GDP-form of the protein may be released from the membrane. It has been known that the PsbO protein dissociates from its docking site upon photoinactivation of PSII electron transport [40]. Recently, it has been reported that the PsbO protein, together with the Mn^{2+} and Ca^{2+} ions of the OEC complex can be reversibly and concomitantly released under non-inhibitory light conditions as well as at pH 6.0 in darkness [15]. In an attempt to investigate the possible role of GTP in the mechanism of its dissociation from the PSII complex, we first studied the release of PsbO from NaCl-washed PSII membranes under similar experimental conditions as used in [15]. Western blots of supernatants and corresponding pellets showed that GTP enhanced the release of PsbO at pH 6.0 during both incubation in darkness and under moderate light (120 μmol photons m^{-2} s^{-1}) in the presence of an electron acceptor (Fig. 4A). At pH 7.4, the release was stimulated under light conditions, and occurred to a similar extent in the absence and presence of GTP.

To study further the effect of GTP on the dissociation of PsbO from the PSII complex under moderate (100 and 200 μmol photons m^{-2} s^{-1}) and high light (1000 μmol photons m^{-2} s^{-1}) inhibitory conditions, we used isolated LHCII–PSII supercomplexes. They have a dimeric organization, retain all the OEC proteins, and show high levels of GTPase activity (data not shown). In this type of preparation, PSII is highly active in oxygen evolution [27], and extremely stable when it comes to PsbO release due to the presence of 1.0 M of glycine betaine in the incubation buffer. GTP stimulated the inactivation of oxygen evolution at all three

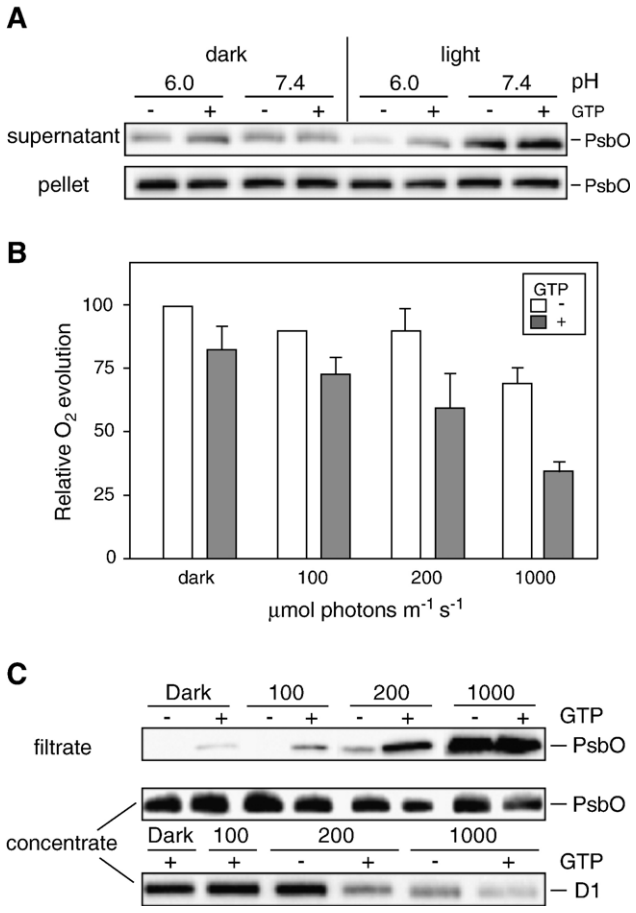

Fig. 4. Effect of pH and light on the GTP-induced release of PsbO and oxygen evolution. (A) Western blots with anti-PsbO antibody of supernatants and pellets obtained from NaCl-washed PSII membranes (0.075 mg of Chl ml^{-1}) incubated for 30 min in darkness or light (120 µmol photons m^{-2} s^{-1}) in the presence of 0.1 mM DCBQ and 0.5 mM ferricyanide at pH 6.0 and 7.4 in the absence (−) and presence (+) of 200 µM GTP. (B) LHCII–PSII supercomplexes (0.2 mg of Chl ml^{-1}) were dark-incubated or illuminated in the absence of acceptors for 30 min in the absence (−) and presence (+) of 200 µM GTP. The extent of PSII inactivation in darkness, at moderate (100 and 200 µmol photons m^{-2} s^{-1}) and high (1000 µmol photons m^{-2} s^{-1}) light intensities is shown as a plot of relative O_2 evolution as compared to untreated samples. The standard deviations were calculated for data obtained from three independent experiments. (C) The amount of released PsbO protein after the same treatments of LHCII–PSII supercomplexes as described in B, was detected by Western blotting in the filtrates. The amounts of remaining PsbO and D1 protein were immunodetected in the corresponding concentrates.

tested light intensities (Fig. 4B). Concomitant with this loss of photosynthetic activity was the GTP-stimulated release of the PsbO protein, as shown by Western blot analyses of the filtrates and the corresponding concentrates (Fig. 4C). At the high light intensity, the PsbO protein dissociated from the inactivated complexes even in the absence of externally added GTP, in accordance with [40]. As a consequence of photoinactivation of oxygen evolution, the D1 protein was degraded in a GTP-stimulated manner, as revealed by Western blot analyses of the concentrates (Fig. 4C). The effect of GTP on the degradation of the D1 protein is particularly obvious at 200 µmol photons m^{-2} s^{-1}, correlating with the largest effect on the release of the PsbO protein.

4. Discussion

The results presented here and previously [17] suggest that the higher plant PsbO protein has the capacity to bind and hydrolyze GTP. This activity is sensitive to light intensity, brings about a release of the PsbO protein from its docking site on the lumenal surface of PSII, and enhances the rate of D1 protein degradation.

Based on spectroscopic measurements, we show that MgGTP binding to PsbO induces specific changes in the secondary and tertiary structure of the protein in solution. However, optimum GTPase activity of the PsbO protein requires binding to the PSII complex and probably the action of regulatory components such as a dimeric organization. GTP binding and hydrolysis readily occur in darkness, but the induced changes may not be sufficient to release the PsbO protein from its docking site on the lumenal surface of PSII. Nevertheless, dissociation of PsbO proceeds under light conditions [15,40], and is considerably stimulated by its GTPase activity.

GTP-binding proteins act as molecular switches cycling between the GDP-bound ('off') and the GTP-bound ('on') state. Upon binding of slowly hydrolyzable GTP analogues, the two states coexist, and the 'off' conformation becomes predominant in the absence of an 'effector' molecule, as recently demonstrated by using NMR spectroscopy [41]. Thus, the fact that neither GDP nor GTPγS induce detectable changes in the secondary structure of PsbO in solution does not mean that they do not bind to the protein, but rather that they may preserve the 'off' conformation of the protein. In support of the existence of a GDP-bound form, it was previously reported the ability of GDP to compete by 50% the radioactive azido-GTP labeling of PsbO [17]. In the same type of experiments, GTPγS competed only by 30% the radiolabeling of PsbO (Spetea, C. and Andersson, B., unpublished observations). Furthermore, GTPγS partially inhibited the hydrolysis of the natural nucleotide (Fig. 3), indicating that it can bind to PsbO, although with lower efficiency than GTP. The somehow different nucleotide-binding properties of PsbO as compared to the classic GTPases, could be explained by the sequence changes in the putative G domains (Fig. 1A).

Most GTPases require the magnesium nucleotide complex as a native substrate. Changing the metal ion (i.e. Mn^{2+} or Ca^{2+} for Mg^{2+}) or leaving it out of the reaction mixture can therefore greatly affect the binding and the enzymatic activity. In the case of classic GTPases such as H-p21-ras, Mn^{2+} increases the pKa of the γ-phosphate group of GTP by 0.5 units, leading to a better efficiency in its extraction/hydrolysis [42]. Our data show that Mg^{2+} is required for binding of GTP to PsbO, since only MgGTP can induce structural changes (Fig. 2). Free Mg^{2+} is present in mM concentration range in the thylakoid lumen [43]. Initial experiments indicate that Ca^{2+} and especially Mn^{2+} ions considerably stimulate the GTPase activity of PsbO in solution as compared to Mg^{2+}. It has been argued that the PsbO protein acts as a 'chaperone' for Mn^{2+} and Ca^{2+} ions released from the OEC into the lumen during exposure to either non-inhibitory or inhibitory light ([15] and references therein). In this work, we

show that GTP stimulates the dissociation of PsbO from PSII under both types of light conditions (Fig. 4). Therefore, we propose that the GTPase activity of PsbO may confer an advantage for the plant in the efficient binding of the released OEC ions. Under physiological conditions, the reversible dissociation of PsbO together with OEC ions is suggested ([15]. Under photoinhibitory conditions, dramatic conformational changes occur within the damaged PSII complex, which together with the GTP-induced changes affect the docking site of PsbO, and induce its irreversible dissociation from PSII. The OEC ions bound to PsbO are then reused for the repair of PSII following photoinhibition.

Due to low intrinsic hydrolysis and nucleotide dissociation rates, the cycling of small GTPases is controlled mainly by the GAP and GEF-type of regulators. Switch I of small GTPases is proposed to interact with the GAP as well as with 'effector' molecules [20]. The corresponding Switch I in PsbO, namely the β1–β2 loop, interacts with CP47 of the other PSII monomer within the dimeric complex, and therefore GTP hydrolysis may initiate the change in the oligomeric organization of PSII. This does not obligatorily mean that GTP hydrolysis leads to the dissociation of PsbO from PSII, since the extended and flexible loop joining β5 and β6 strands (β5–β6), mainly involved in interaction with the lumenal surface of PSII complex [20], is quite far from the postulated GTP-binding region of the protein. In addition to GTP (Fig. 4), other factors such as light [40] and metal ions [15] are required to induce PsbO dissociation from PSII. Other extrinsic proteins such as PsbP and PsbQ may act as GDI components, preventing the nonspecific release of PsbO and the bound nucleotide from the lumenal surface.

Plant PsbO plays an essential role in the degradation of the D1 protein [44,45], and previous *in vitro* studies demonstrated a requirement of GTP for the degradation step [18,19]. The findings of this work bring experimental support for the function of PsbO as a GTPase in the light-mediated D1 turnover. Two different proteases were reported to be involved in D1 degradation in plants, namely DegP2 and FtsH, both located in the stroma-exposed regions of the thylakoid membrane [46,47]. There is no documented interaction between PsbO and either of the above proteases, which act on the D1 protein from a side of the membrane opposite to the one where PsbO binds to PSII. Therefore, based on the predicted location of G domains and flanking Switch regions, we suggest that the GTPase activity of PsbO is involved in repair events occurring in the grana regions of the thylakoid membrane, such as the monomerization of damaged PSII complexes, which is a prerequisite step for their migration to the stroma lamellae, D1 degradation and *de novo* synthesis [48]. This possibility requires further investigation.

The structure of a well-studied canonical GTP-binding protein, named H-ras-p21, is a hydrophobic core consisting of six β-strands connected by hydrophilic loops and five α-helices, with the G domains found in loops located on the same side of the protein [22]. Except for the partially conserved G domains, the amino acid sequence and the structural homology model of spinach PsbO [6] are quite different from those of H-ras-p21, and the GTP-binding site is predicted inside the β-barrel domain of the protein. We, therefore, suggest that PsbO may represent a novel plant-specific GTPase, with significant structural differences from the classic GTPases. Since it shares several unusual features with the Toc34 protein [34,35], we propose that it belongs to the septin family of GTPases [23].

In addition to the prediction data for G domains, we have several experimental indications that PsbO-like GTP-binding proteins only occur in higher plants. The PsbO protein was not photolabeled with radioactive azido-GTP in *Synechocystis* or *Thermosynechococcus* thylakoids (Spetea, C. and Andersson, B., unpublished observations) in contrast with the results obtained using spinach preparations [17]. No stimulation by GTP of D1 protein degradation was detected in photoinactivated *Synechocystis* thylakoids (Spetea, C., Kanervo, E., Aro, E.-M., Andersson, B., unpublished observations) as compared to spinach thylakoids [18]. Therefore, the proposed GTPase activity of PsbO may only occur in higher plants as an early and tightly regulated event for optimizing the function of OEC and efficient turnover of the PSII reaction centre D1 protein.

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