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The 33 kDa subunit of photosystem II is a GTP-binding protein – presence of nucleotides in the thylakoid lumen

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Introduction

Recently, we reported in Spetea et al. (1999) that following photoinactivation of photosystem II (PSII), proteolysis of the reaction center D1 protein proceeds in at least two enzymatic steps with different nucleotide requirement. The primary proteolytic step is GTP-dependent whereas the secondary step requires ATP. The primary step was found to be associated with isolated PSII complexes. Binding of GTP to the thylakoid membrane is tight and light-dependent. In Spetea et al. (2000), we demonstrated that the role of GTP in D1 protein degradation is at the level of the proteolytic enzymes, directly or via a chain of reactions. The concept of a more general significance for the existence of GTP tightly bound to the thylakoid membrane for regulatory reactions during photosynthesis has also been raised. In this work, we have investigated the presence of GTP-binding proteins associated with the thylakoid membrane.

Materials and methods

Spinach (*Spinacea oleracea*) leaves were used for the isolation of thylakoid membranes and PSII core complexes (Spetea et al., 1999). The thylakoids and PSII cores were finally suspended in 50 mM HEPES-NaOH (pH 7.4) containing 400 mM sucrose, 5 mM $MgCl_2$ and 15 mM NaCl, supplemented with 5 mM $CaCl_2$ and 0.01 % (w/v) dodecyl maltoside for the cores.

After dilution in the same buffer to a final Chl concentration of 0.3 mg/ml, samples were illuminated in an Elisa plate (50 µl final volume) with heat-filtered visible light (1500 µmoles of photons/m^2/s) for 30 min on ice. Where indicated, 50 µM DCMU was added at the onset of illumination. Dark-control or pre-illuminated samples were incubated with [α-^{32}P]8-N_3GTP (8 Ci/mmol, ICN Pharmaceuticals) on ice for 3 min, and then irradiated for 90 sec with a handheld 254-nm UV lamp.

PSII cores photolabeled with [α-^{32}P]8-N_3GTP were washed with 0.8 M Tris-HCl (pH 8.5) for 30 min on ice in darkness and the pellet was separated from the supernatant in Centricon tubes (100 kDa cut-off filter).

SDS-PAGE and western blotting were performed as previously described (Spetea et al., 1999). The photolabeled proteins were detected by phosphoimaging. The OEC33 protein was identified using an antiserum raised in rabbit against the spinach protein.

Results

Photoaffinity labeling of a 33 kDa protein with [α-^{32}P]8-N_3GTP. It was of interest to perform GTP-labeling experiments in thylakoids and PSII cores in an effort to identify potential GTP-binding proteins with possible involvement in the process of D1 protein degradation as well as other regulatory reactions in photosynthesis. Dark-control and 30 min pre-illuminated samples were photolabeled with [α-^{32}P]8-N_3GTP, which covalently binds with high affinity at specific sites following UV-activation. Photolysis of [α-^{32}P]8-N_3GTP in thylakoids (panel *A*) revealed labeling of two bands with M_r of 36.5 and 33 kDa, respectively (**Fig. 1**). The labeling of the 36.5 kDa band was enhanced by pre-illumination (*lane 4*) as compared to the dark-control (*lane 2*), whereas the 33 kDa band could only be detected in the pre-illuminated samples (*lane 4*). Following photolabeling of PSII cores (panel *B*) only the 33 kDa band could be detected. The binding was four-fold stronger in pre-illuminated cores (*lane 8*) as compared to the dark-control ones (*lane 6*), and ten-fold stronger than in pre-illuminated thylakoids (*lane 4*).

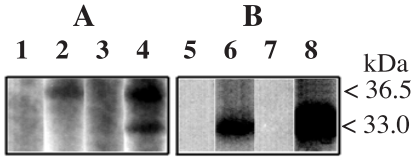
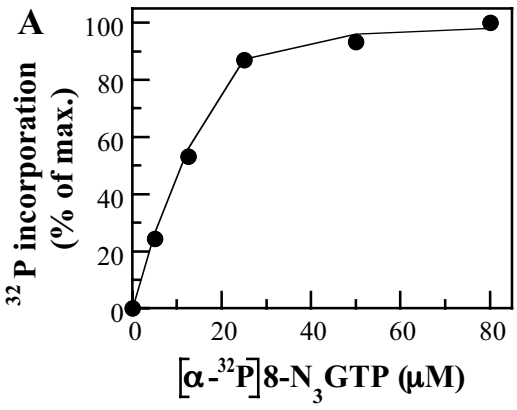


Fig. 1. Photoaffinity labeling of thylakoids and PSII core complexes with [α-^{32}P]8-N_3GTP. Thylakoids (*A*) and PSII core complexes (*B*) were dark-incubated (*lanes 1, 2, 5* and *6*) or pre-illuminated (*lanes 3, 4, 7* and *8*) on ice for 30 min, followed by addition and incubation with 25 μM [α-^{32}P]8-N_3GTP for 3 min on ice in darkness. Only samples from *lanes 2, 4, 6* and *8* were photolysed for 90 sec on ice, whereas the others were kept in darkness for the same period of time and subjected to SDS-PAGE and phosphoimaging.

These results suggest the following: (*i*) the 33 kDa, but not the 36.5 kDa protein, is a PSII component; (*ii*) the 33 kDa protein is not readily accessible to GTP in dark-control thylakoids possibly due to location on the lumenal side of the membrane; (*iii*) labeling of both 36.5 and 33 kDa is light-dependent; (*iv*) as it will be further analyzed (Spetea et al., these Proceedings *S24-005)*, the 36.5 kDa protein could be a light-dependent trans-thylakoid transporter of GTP (and other nucleotides).

Photolabeling with [α-^{32}P]8-N_3GTP of the 33 kDa band is specific. To show the specificity of [α-^{32}P]8-N_3GTP interaction with the 33 kDa band, photolabeling of the azidonucleotide should exhibit UV light dependency and saturation effects. The UV light dependency is demonstrated in **Fig. 1*A***. Under the experimental conditions described, photolabeling of the 33 kDa band was saturated at about 40-50 μM probe (**Fig. 2*A***). The apparent K_m of this interaction calculated from Scatchard plots of the data was 22.8 μM, which satisfies a major criterion for specificity of nucleotide interaction. To further define the nature of the binding sites, the effects of various nucleotides on the photolabeling of the 33 kDa band were compared in **Fig. 2*B***. Like GTP, GDP reduced the photolabeling of the 33 kDa band by approx. 50 %. GMP and other nucleotides did not compete or rather stimulated the binding of [α-^{32}P]8-N_3GTP.



B

Nucleotide	none	GTP	GDP	GMP	ATP	CTP
% of control	100	52.3	54.6	98.0	110.0	108.0

Fig. 2. Photolabeling with [α-^{32}P]8-N_3GTP of the 33 kDa band in PSII core complexes is specific.*A*, *Saturation*. PSII core complexes were pre-illuminated and photolysed with the indicated concentrations of [α-^{32}P]8-N_3GTP followed by SDS-PAGE and phosphoimaging. *B*, *Competition.* The photolabeling of the 33 kDa protein with 25 μM [α-^{32}P]8-N_3GTP was carried out as described in *A*, but in the presence of 250 μM various cold nucleotides. Values represent the percentage of remaining photolabeling compared to the control values obtained in the absence of cold nucleotides.

Identification of the GTP-binding protein as the PSII OEC33 subunit. In order to identify the 33 kDa GTP-binding protein, PSII cores pre-illuminated and subsequently photolabeled with [α-^{32}P]8-N_3GTP, were subjected to alkaline Tris-washing and analyzed by SDS-PAGE and phosphoimaging. As shown in **Fig. 3*A*,** the radioactivity was mostly recovered in the supernatant containing the soluble proteins (*lane 3*) as compared to the pellet (*lane 2*). The Coomassie-stained gel revealed a single band of 33 kDa in the supernatant (not shown). Since the 33 kDa subunit of the oxygen-evolving complex (OEC33) is well known to be released by Tris-washing, western blotting with anti-OEC33 was carried out. As shown in **Fig. 3*B***, the immuno-decorated band followed the same pattern as the radioactive one. Furthermore, sequence analysis of the Coomassie-stained 33 kDa band indicated that the first seven amino acids EGGKRLT correspond to the amino terminus of the mature OEC33 from spinach.

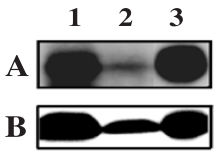


Fig. 3. Identification of the GTP-binding protein as the PSII OEC33 protein. PSII cores were pre-illuminated and photolabeled with 50 μM [α-^{32}P]8-N_3GTP as described in **Fig. 1** followed by Tris-washing, centrifugation and separation by SDS-PAGE of the proteins contained in the pelleted PSII complexes and the supernatant. *A*, phosphoimage of the Coomassie-stained gel in the region around 33 kDa; *B*, Western blot with anti-OEC33 antibody of photolabeled PSII cores (*lane 1*), pellet (*lane 2*) and supernatant (*lane 3*).

In Spetea et al. (2000) we reported that GTP does not stimulate the light-induced D1 protein degradation in the presence of herbicides, known to bind at the quinone site of the D1 protein and thus affect the accessibility of the protease to the cleavage site. The presence of DCMU during illumination of PSII cores inhibited the labeling of the OEC33 protein with [α-^{32}P]8-N_3GTP. The photolabeling reached the same level as in the dark-control (**Fig. 4**). This result together with the light effect (**Fig. 1*B***) suggest a change in the affinity of the OEC33

protein for GTP binding, which is dependent upon electron transport possibly *via* redox-sensing.

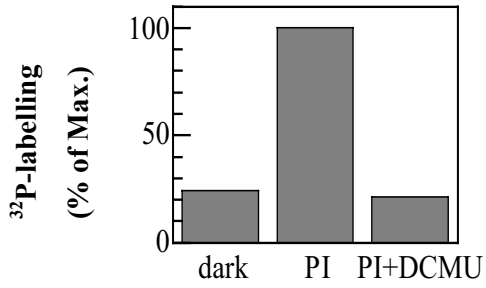


Fig. 4. Effect of DCMU on the photolabeling of the OEC33 protein in PSII core complexes. Pre-illumination (PI) of PSII core complexes was carried out in the absence or presence of 50 μM DCMU followed by photolabeling with 25 μM [α-^{32}P]8-N_3GTP and SDS-PAGE. The level of photolabeling in dark-control PSII cores is also shown.

Discussion

The OEC33 protein is the product of *psbO* gene present in all oxygen-evolving photosynthetic organisms from cyanobacteria to higher plants and is important in maintaining the structural integrity of the manganese cluster, which catalyzes the water-splitting reaction (Bricker and Frankel, 1998). The spinach OEC33 protein does not contain typical GTP-binding motifs, like in the case of the GTP-binding protein tubulin (PROSITE PS00017). The saturation and competition studies performed with [α-^{32}P]8-N_3GTP indicate that the binding of GTP to this PSII protein is specific. Previously, Gal et al. (1996) reported that isolated OEC23 protein is able to bind both GTP and ATP, but the relevance of this finding has not been further elucidated. The discovery of GTP binding to the OEC33 protein points to the existence of nucleotides in the thylakoid lumen and raises several crucial questions, *e.g.* the presence of a nucleotide transport system across the thylakoid membrane (see Spetea et al., these Proceedings *S24-005*). However, another challenge is to understand the fundamental significance of nucleotide metabolism in the thylakoid lumen. Here we speculate that the PSII OEC33 subunit, in addition to the role of a manganese-stabilizing protein, has a potential function as the α-subunit of a G-protein complex together with OEC23 (β-subunit?) and OEC16 (γ-subunit?) proteins. Recent results indicate that following GTP-binding, the OEC33 protein can change its binding affinity for PSII complex (Eshaghi, 2001).

Acknowledgments

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