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Phosphorylation of spinach chlorophyll-protein complexes

CPII*, but not CP29, CP27, or CP24, is phosphorylated in vitro

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Previous studies have indicated that the reversible phosphorylation of a population of antenna complexes that can donate energy to PS II ('mobile LHC II') plays a regulatory role in the state 1–state 2 transition in thylakoid membranes. The relationship of phosphorylated LHC II to the multiple PS II-associated chlorophyll *a/b*-proteins resolvable on green gels is currently unclear. We have used a high resolution gel system to analyze thylakoids phosphorylated in vitro. The only PS II-associated antenna complex to become phosphorylated is CPII*, indicating that this complex represents the mobile LHC II. The other putative PS II antenna complexes, CP29, CP24, and the new complex designated CP27 which comigrates with CPII, are not phosphorylated and are probably components of the bound 'LHC II' antenna.

Thylakoid; State 1–state 2 transition; LHC II; CP29; Protein phosphorylation; Chlorophyll *a/b*-light harvesting complex; (Spinach)

1. INTRODUCTION

Higher plants are capable of regulating the distribution of excitation energy between photosystem I (PS I) and photosystem II (PS II) to ensure optimal photosynthetic rates under changing environmental conditions. This process, known as the state 1–state 2 transition, is apparently mediated by reversible phosphorylation of the chl *a/b*-light harvesting complex (chl *a/b*-LHC), the major antenna complex of PS II. The current model says that conditions favoring excitation of PS II lead to activation of a thylakoid-bound kinase which phosphorylates a population of chl *a/b*-LHC molecules ('mobile LHC II'). The phosphorylated complexes then migrate from the PS II-enriched grana regions to the PS I-rich stromal membranes, effectively increasing the absorptive cross-section for the PS I reaction center [1,2].

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Under nondenaturing electrophoretic conditions ('green gels'), the chl *a/b*-LHC has been associated with two major bands: an oligomeric form, CPII* (also called LHCP[1] or chl *a/b*-P2**), and the monomer CPII (LHCP[3] or chl *a/b*-P2) [3–5]. Both bands can be phosphorylated in vitro, and fractionation studies have shown that these chlorophyll-proteins and their constituent polypeptides are enriched in stromal membrane fractions following membrane phosphorylation [6–10].

In recent years, improvements in electrophoretic techniques have resulted in the discovery of at least two new chl *a/b*-proteins putatively identified as components of the PS II antenna [5,11–13]. These complexes, CP29 and CP24, migrate in the region of CPII on green gels. In addition, the CP29

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apoprotein has a similar molecular mass to the polypeptide components of the chl *a/b*-LHC. Thus, previous studies have not addressed the question of whether CP29 or CP24 could also be phosphorylated and possibly play a regulatory role in energy distribution. In these experiments, we have used a gel system which provides good resolution of CPII, CP29 and CP24 to ask which complexes are phosphorylated in vitro. Our results show that neither CP29 nor CP24 is phosphorylated, suggesting these complexes are components of the 'bound' PS II antenna. In addition, although CPII* is heavily phosphorylated, there is little phosphorylation of the CPII region. Our results suggest that the CPII region on nondenaturing gels contains another chl-protein complex, which we call CP27, which is also not phosphorylated in vitro.

2. MATERIALS AND METHODS

Thylakoids were isolated from commercially grown spinach by first homogenizing leaves in 0.3 M sorbitol, 0.1% BSA; 5 mM $MgCl_2$, 10 mM NaCl, 50 mM Tricine-NaOH, pH 7.8. The homogenate was filtered through 8 layers of cheesecloth and chloroplasts were pelleted at 1500 $\times$ *g* for 1 min. The pellet was washed in 5 mM $MgCl_2$, 10 mM NaCl, 15 mM Tricine-NaOH, pH 7.8, then resuspended in phosphorylation buffer (0.1 M sorbitol, 5 mM $MgCl_2$, 10 mM NaCl, 1 mM benzamidine-HCl, 5 mM ϵ-aminocaproic acid, 50 mM Tricine-NaOH, pH 7.8) to a concentration of 200 μg chl/ml; chl concentration was determined as described by Arnon [14].

Phosphorylation was initiated by adding 200 μM ATP including 50–100 μCi [γ-^{32}P]ATP (10–30 Ci/mM) and 10 mM NaF, and illuminating the sample with a microscope lamp (500 $\mu E/m^2$ per s) [15]. After 7 min, the membranes were rapidly pelleted, washed 2 $\times$ with 1 mM NaF, 2 mM Tris-maleate, pH 7.0, and suspended in 0.88% octyl glucoside, 0.22% SDS, 10 mM NaF, 40% glycerol, 2 mM Tris-maleate, pH 7.0, such that the ratio of octyl glucoside/SDS/chl = 20:5:1. The sample was incubated on ice for 2 min and solubilized material was electrophoresed on nondenaturing gels as described [11]. Following electrophoresis, the gels were dried and exposed for autoradiography. Alternatively, the green bands were cut from the gel, heated to 90°C for 2 min in 4% SDS, 4% β-mercaptoethanol, 20% glycerol, 0.13 M Tris-HCl, pH 6.8, and re-electrophoresed using a gel system modified from Laemmli [16], containing a 10–17% acrylamide gradient and 4 M urea.

3. RESULTS

Fig.1a shows the chl-protein complexes of phosphorylated spinach thylakoids as resolved by

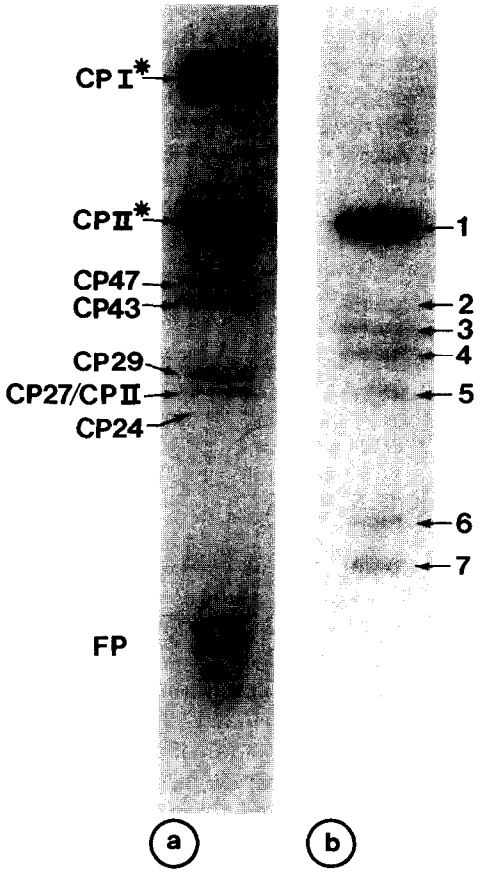

Fig.1. (a) Unstained, mildly denaturing gel showing the chl-protein complexes of ^{32}P-phosphorylated spinach thylakoids. CPI* is associated with PS I, and CP43 and CP47 are probably components of the PS II core complex. CPII*, CP29 and CP24 are PS II antenna complexes; the CPII region contains both monomeric chl *a/b*-LHC complexes (from dissociation of CPII*) plus a new chl-protein designated CP27. See text for discussion. FP, free pigment. (b) Autoradiogram of the gel in (a).

electrophoresis under mild conditions in the presence of octyl glucoside; the bands are labeled using the nomenclature of Camm and Green [5] as modified by Dunahay and Staehelin [11] and are identified in the figure legend. CP24 is just barely seen below CPII; this complex is enriched in PS II-enriched grana membrane preparations and is usually difficult to resolve in whole thylakoid membrane extracts [11]. An autoradiogram of this gel is shown in fig.1b. Seven bands are labeled with ^{32}P, three of which (bands 1, 2 and 5) comigrate with green chl-protein bands seen in fig.1a. The most heavily labeled band is CPII* (band 1). There is some label migrating with CPII (band 5), but little or no detectable phosphorylation of CP29 or of the CP24 region below CPII. There is also some label comigrating with CP43, and with four polypeptides which do not comigrate with visible chl-protein complexes (bands 3, 4, 6 and 7).

The polypeptide composition of phosphorylated spinach thylakoids and of the CP29, CPII, CPII*, and CP24 bands cut from green gels is seen in fig.2a. CPII* contains three polypeptides between 24 and 27 kDa which are typically associated with the chl *a/b*-LHC [1,17]. There is also a high molecular mass band of 54 kDa which probably corresponds to an unrelated polypeptide that comigrated with the excised CPII* band since it is not phosphorylated (fig.2b). In contrast, CP29 contains a major polypeptide of 25.5 kDa which sometimes appears as a doublet and which does not comigrate with the polypeptides of CPII*, plus a minor band at 22.5 kDa. Surprisingly, the CPII band did not contain the three polypeptides found in CPII*. Instead, the CPII band contains a single polypeptide of 26 kDa which also does not comigrate with the CPII* components, plus a lower molecular mass band in the position of the 22.5 kDa band found in CP29. CPII has been widely reported to be a monomeric form of CPII*, and re-electrophoresis of CPII* under mild conditions produces a green band which migrates in the position of CPII [5,18,19]. Therefore, it appears that under the conditions used here, the chl *a/b*-LHC is stabilized almost entirely in the form of CPII*. This allows the resolution of another green band, which we call CP27 (see section 4), which migrates in the position of CPII but contains a unique set of polypeptides. CP24 contains three 20–23 kDa polypeptides as reported [11].

Fig.2b shows the autoradiogram of the gel in fig.2a. In phosphorylated thylakoids, the radioactive label is found primarily in two polypeptides which comigrate with the two higher molecular mass bands of the chl *a/b*-LHC found in CPII*. This allows the resolution of another green band, which we call CP27 (see section 4), which migrates in the position of CPII but contains a unique set of polypeptides. CP24 contains three 20–23 kDa polypeptides as reported [11].

Fig.2b shows the autoradiogram of the gel in fig.2a. In phosphorylated thylakoids, the radioactive label is found primarily in two polypeptides which comigrate with the two higher molecular mass bands of the chl *a/b*-LHC found in CPII*. This is confirmed by re-electrophoresis of CPII*. In contrast to CPII*, no label is found associated with the polypeptides of CP24 or in the CP29 apoprotein. The phosphorylation pattern seen in CPII resembles that of CPII*, although the two complexes contain different polypeptides based on Coomassie staining. Thus, if there is a second chl-protein complex in the CPII region (CP27) which is not a CPII* monomer as suggested above, this new complex is apparently not phosphorylated and the small amount of label seen in this region on green gels (fig.1b) results from some dissociation of phosphorylated CPII* complexes.

There are two interesting features of the phosphorylation pattern of the CPII* polypeptides. First, only two of the three components of CPII* are phosphorylated, although all three proteins are immunologically related [20,21]. Second, the smaller of the two phosphorylated CPII* components is apparently labeled with a higher specific activity than the higher molecular mass band; both bands are of approximately equal density in the autoradiogram although the larger component stains more intensely with Coomassie blue. This result is in agreement with several recent studies reporting differential phosphorylation and migration of the chl *a/b*-LHC polypeptides [8,22].

As reported by other laboratories, phosphorylation of thylakoids results in the labeling of several polypeptides in addition to the components of the chl *a/b*-LHC [15,22]; this is seen more clearly in the more heavily loaded lane 1 in fig.2b. These include minor bands at 60 kDa and 39 kDa, a doublet between 30 and 32 kDa, and two low molecular mass proteins below 14 kDa. Re-

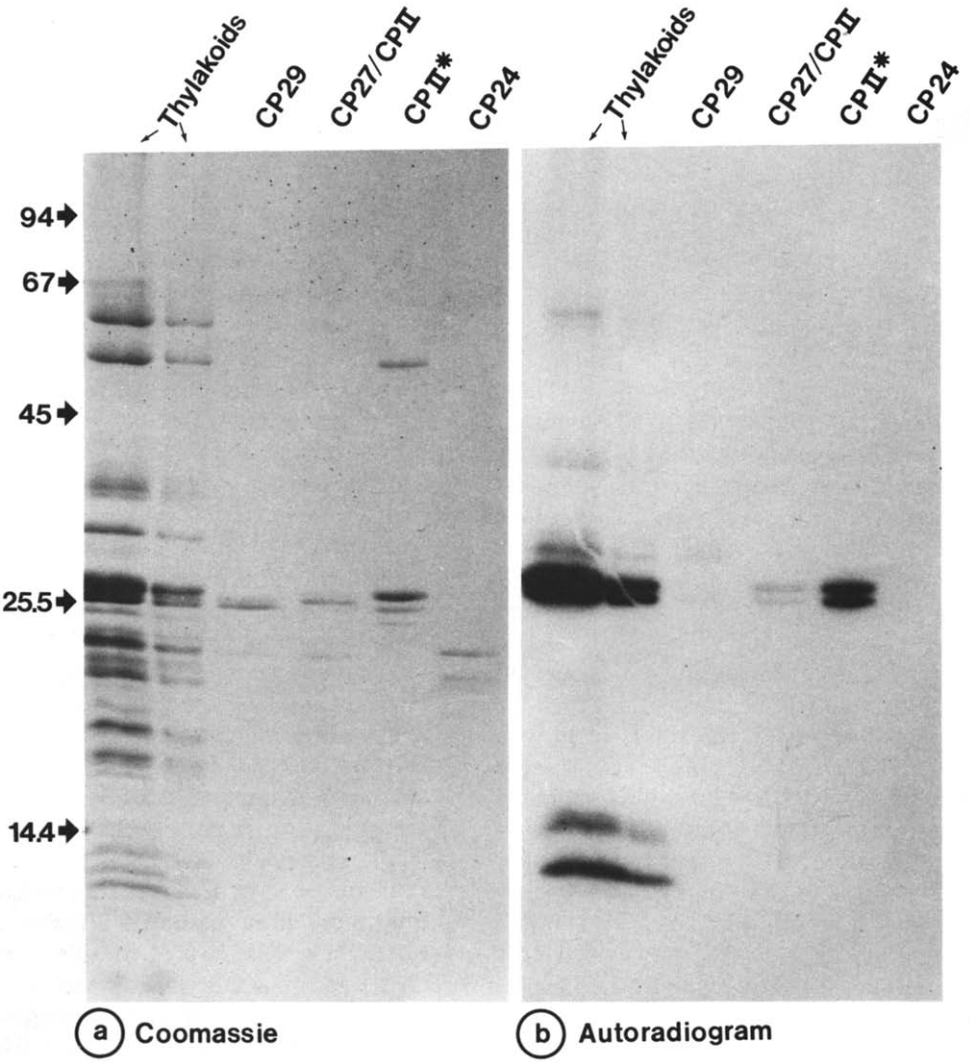

Fig.2. (a) Polypeptide composition of phosphorylated spinach thylakoids and chl-protein complexes isolated as green bands from the gel seen in fig.1a. Lanes 1 and 2 are two different loadings of identical samples (10 μg and 2 μg chl, respectively). See text for discussion. The molecular mass standards (in kDa) are indicated by arrows and are (top to bottom) phosphorylase *a*, bovine serum albumin, ovalbumin, chymotrypsinogen and lysozyme. Note: this somewhat lightly loaded gel was chosen for illustrative purposes because of its superior resolution. Heavier loaded gels (not shown) did not display any additional bands either after Coomassie blue staining or in autoradiograms. (b) Autoradiogram of the gel in (a).

electrophoresis of other gel bands from a green gel of phosphorylated thylakoids as in fig.1a showed that the 39 kDa protein is associated with CP43 (band 2 from fig.1a), bands 3 and 4 represent the phosphorylated 30 kDa doublet, and bands 6 and 7 represent the two low molecular mass bands seen in fig.2b (not shown). The 60 kDa phosphorylated polypeptide could be the LHC II kinase [24] or the β-subunit of the CF1 ATP synthase.

4. DISCUSSION

The antenna complex which directs excitation energy to the PS II reaction center known as

LHC II is apparently composed of several distinct chl *a/b*-protein complexes. We have demonstrated that incubation of spinach thylakoids in the light in the presence of [^{32}P]ATP results primarily in the phosphorylation of the major PS II antenna known as the chl *a/b*-LHC, represented on green gels as CPII*. We believe that the chl *a/b*-LHC is equivalent to the mobile LHC II complex which mediates the state 1–2 transition [1,2]; this complex is not tightly bound to PS II, is capable of migration from granal to stromal membranes upon phosphorylation, and forms 8 nm PF particles seen in freeze-fractured membranes [10,25]. In contrast, CP29, CP24, and CP27, also identified as PS II-associated antenna complexes [5,11,12], are not phosphorylated under the conditions described here and are probably components of the bound LHC II. These complexes are associated with the PS II reaction center and are most likely components of the large EF freeze-fracture particles.

The results presented here are in agreement with previous studies from our laboratory in which we used polyclonal antibodies specific for CPII* and CP29 to localize these complexes in phosphorylated and non-phosphorylated thylakoid membranes [20]. Using immunogold labeling of fixed and embedded membranes we found that about 80% of both CPII* and CP29 are localized in grana membranes in nonphosphorylated thylakoids. Upon phosphorylation, the proportion of CPII* in the stroma membranes increased from 20% to about 50%, while CP29 remained primarily in the grana membranes.

The data presented here supporting the existence of a new chl-protein complex which comigrates with CPII are in agreement with a recent report by Bassi and Simpson [12]. These authors reported that solubilization and electrophoresis of PS II-enriched grana membranes in the presence of octyl glucoside plus 40% glycerol produced essentially complete stabilization of CPII*. These conditions allow the resolution of the CPII region into three chl-protein bands which are apparently analogous to the CP29, CP27, and CP24 complexes described here. We have designated the new chl-protein band which comigrates with CPII 'CP27' as suggested by Dr Beverly Green at the VIIth International Congress on Photosynthesis in August, 1986. The function of CP27 and its relationship to the other chl *a/b*-protein complexes is not known, although Bassi and Simpson [12] have proposed a model of PS II organization which includes CP27 as a non-phosphorylated component of the PS II antenna. Preliminary experiments in our laboratory using polyclonal antibodies specific for CPII* and CP29 to probe the polypeptides of CP27 were inconclusive; both anti-CP29 and anti-CPII* recognized polypeptides associated with the new complex, although CPII* crossreactivity could result from a small amount of comigrating CPII monomer polypeptides.

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