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FUNCTIONAL DISCRIMINATION BETWEEN PHOTOSYSTEM-II ASSOCIATED CHLOROPHYLL *a* PROTEINS IN *ZEA MAYS*

KENNETH J. LETO

Central Research and Development Department, E.I. du Pont de Nemours and Company, Wilmington, DE 19898 (U.S.A.)

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Three minor Chl *a* proteins were detected in electrophoretic profiles from wild-type maize thylakoids. The spectral characteristics of these Chl proteins and the apparent molecular weights of their constituent apoproteins suggested that they were associated with the Photosystem-II reaction center. One of these Chl *a*-proteins, CPa-1, was present in wild-type thylakoids and a photochemically active Photosystem-II particle, but was missing from thylakoids of a mutant-lacking Photosystem-II reaction center. CPa-2, on the other hand, was enriched in mutant thylakoids but was completely missing from the Photosystem-II particles. We conclude that CPa-1 is most likely to contain the photoactive chlorophyll of Photosystem II, while CPa-2 is not required for Photosystem-II activity. The apparent molecular weights of the major CPa-1 and CPa-2 apoproteins were 48000 and 42000, respectively. The third minor Chl protein seems most likely to be an electrophoretic variant of CPa-1 and has been designated CPa-1*. Seven other Chl proteins were detected in wild-type profiles. Many of these Chl proteins appeared to be oligomers or highly order complexes of LHCP and CP-1.

Introduction

Since the initial resolution of CP-I and CP-II on polyacrylamide gels [1,2], advances in electrophoretic technique and the use of progressively gentle solubilization procedures have led to the detection of Chl proteins in increasing number. In the most highly resolving systems 10–12 Chl proteins can be visualized [3,4], many of which appear to be oligomers or higher order complexes of CP-I and CP-II [3–10]. It is presently agreed that CP-I contains P-700, the photoactive chlorophyll of Photosystem I [10,11], while CP-II (also called the LHCP), contains both Chl *a* and Chl *b* and serves as an antenna complex primarily associated with Photosystem II. The oligomers of these complexes may represent increasingly native forms of the Chl proteins as extracted from the thylakoid [5,12]. Together, CP-I, LHCP and their oligomers comprise up to 75% of the chlorophyll remaining complexed to protein after electrophoresis [5,6,10,13–15].

In addition to CP-I, LHCP and their oligomers, a minor 30–55 kDa Chl *a* protein has frequently been observed. This Chl *a* protein, originally termed Complex IV [16] and also called CPa [3,5,6], A [17], or the A-2 complex [18,19], was thought to be associated with the Photosystem-II reaction center on the basis of genetic and developmental evidence [6,8,9,16,18–22]. Recently, two minor Chl *a* proteins with apparent molecular weights of

Abbreviations: Chl, Chlorophyll; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DCBQ, 2,6-dichloro-*p*-benzoquinone; DCIP, 2,6-dichlorophenolindophenol; PS II, Photosystem II; DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea.

40 000–43 000 and 47 000–50 000 have been resolved in the region commonly occupied by CPa [3,4,7,13,23,24]. In *Chlamydomonas*, the apoproteins comprising these complexes are immunologically unrelated, and both are lost from the thylakoid membrane in nuclear mutants lacking Photosystem-II reaction centers [22,23]. This indicates that the two Chl *a* proteins are distinct, and suggests an association of both of them with Photosystem II.

Despite these correlations, the association of the two minor Chl *a* proteins with the Photosystem-II reaction center remains unclear. First, the loss of Photosystem II is not obligatorily associated with the loss of these Chl *a* proteins, since several maize and barley mutants exhibiting drastic reductoins in Photosystem-II activity nevertheless contain appreciable amounts of at least one of these species [3,19]. Second, it is not clear which of the two Chl *a* proteins is most likely to contain the photoactive Chl of Photosystem II, although the most recent evidence suggests that the slower migrating species (CPa-1) is the most likely candidate [25]. Finally, it is not known whether one of these Chl *a* proteins alone is sufficient to support Photosystem-II activity, or whether both are required.

In the present study we have been able to make a functional distinction between the minor Chl *a* proteins by comparing polypeptide and Chl *a* protein profiles of wild type maize with profiles obtained from a fully green Photosystem-II deficient maize mutant and from a highly resolved Photosystem-II particle. We find that the loss of CPa-1 in the Photosystem-II mutant is specific, and that Photosystem-II particles retain activity in the complete absence of the faster migrating Chl *a*-protein (CPa-2). In addition, we have identified a third minor Chl *a* protein which appears to be an electrophoretic variant of CPa-1. Most of the other Chl proteins appearing in the maize profile have been characterized with respect to polypeptide composition and identified as oligomers of CP-I or the LHCP.

Materials and Methods

Plant material

Maize (*Zea mays* L.) stock segregating the recessive nuclear mutation *hcf**-3 was derived from an original accession (Neuffer E-846) by two cycles of crossing onto vigorous hybrid stock followed by propagation to the subsequent F_2 or F_3 generation. Seedlings were screened for elevated levels of chlorophyll fluorescence as described [27]. The genetic [28] and photosynthetic [21,29,30] characteristics of this mutant have been reported previously. Seedlings were grown to the three leaf stage during a 16 h (28°C during the day, 10°C at night) photoperiod in a controlled environment chamber under daylight fluorescent and incandescent illumination, totalling 1000–1200 $\mu E \cdot m^{-1} \cdot S^{-1}$.

Chloroplast isolation

Chloroplasts were isolated from seedlings by grinding 10 g of leaf material in a Waring Blendor with 100 ml of ice-cold grinding medium containing 0.4 M sorbitol/0.1 M sodium-tricine (pH 7.8)/10 mM NaCl/5 mM $MgCl_2$/5 mM Na-EDTA (pH 7.8)/5 mM Na-ascorbate (pH 7.8)/0.25% (w/v) BSA (Fraction V)/100 mg solid polyvinylpolypyrrolidone. The brei was filtered through eight layers of cheesecloth and a pellet collected by centrifugation at 4080 × *g* for 10 min in a swinging bucket (Sorvall HB-4) rotor. The chloroplasts were osmotically shocked and unstacked by washing three times in 10 mM sodium-tricine (pH 7.8) and 10 mM NaCl. The final pellet was resuspended in washing buffer at a concentration of 1.5 mg Chl/ml as determined by the method of MacKinney [31].

Polyacrylamide gel electrophoresis

Gradient polyacrylamide gel electrophoresis was performed using the gel and buffer formulations described by Delepelaire and Chua [23]. For routine analysis of Chl proteins, slab gels (analyzing gel 13 × 14 cm, 1.5 mm thick, 2 cm stacking gel) were run in the dark at 2–4°C in a thermostatted apparatus (Protean slab cell, Bio-Rad) at a constant power setting of 40 W (two slabs) for 3–5 h, or until the minor Chl *a* proteins were clearly resolved. Thylakoid membranes (two volumes) were solubilized on ice for 5 min or less in one volume of a buffer containing 150 mM Na-tricine (pH 7.8)/150 mM dithiothreitol/21% (w/v) glycerol/0.003% (w/v) bromophenol blue/6% (w/v) lithium dodecyl sulfate to give a final Chl

concentration of 1 mg/ml at a detergent : Chl ratio of 20 : 1 (w/w).

In some instances Chl proteins were separated preparatively by loading 1–1.5 ml of solubilized membranes onto a stacking gel cast without wells, and electrophoresis carried out as described above. Further purification was accomplished by excising a gel strip containing the Chl protein of interest and subjecting it to a second cycle of electrophoresis. Proteins were recovered after the second cycle by electroelution during analytical electrophoresis or by homogenization of gel slices in 10 mM sodium-tricine (pH 7.8) and 10 mM NaCl. In the latter case the slurry was clarified by centrifugation and the supernatants concentrated in a vacuum centrifuge.

Analysis of lamellar polypeptides and proteins recovered from gel slices was performed on 0.75 mm or 1.5 mm thick slab gels as outlined above. Membranes were dissolved as outlined above or in the solubilization buffer described by Laemmli [30]. Proteins were stained as in Ref. 21 followed, where indicated, by overstaining with a commercial silver stain (Bio-Rad). Apparent molecular weights were determined using phosphorylase *a* (92 000), bovine serum albumin (68 000), ovalbumin (45 000), carbonic anhydrase (30 000), chymotrypsinogen (25 500), soybean trypsin inhibitor (21 500), lysozyme (14 400), and cytochrome *c* (12 400) as standards.

Fluorescence from Chl proteins was excited by back illumination with hand held long wavelength ultraviolet source.

Proteolytic mapping

Mapping of polypeptides by partial proteolysis with *Staphylococcus aureus* V_8 protease during electrophoresis was performed as described by Cleveland et al. [33] with the following modifications: SDS was replaced by LDS in the upper running buffer and stacking gel; detergent was omitted from the analyzing gel and lower running buffer; and EDTA (1 mM) was included in the upper running buffer only. The gel was comprised of a 5% (w/v in acrylamide), 5 cm stacking and a 16 cm 9–17% analyzing gel. Excised gel slices were prepared for analysis as described in [33]. Gels were run at 70 W, 20°C; the run was interrupted for 30 min to allow digestion of eluted proteins when the tracking dye entered the analyzing gel.

Other methods

Absorption spectra of Chl proteins in gel slices were measured using a Beckman DU-8 spectrophotometer. DCIP photoreduction was measured in a Cary 17 spectrophotometer modified for side illumination. Oxygen uptake and evolution were measured at 22°C using a Hansatech oxygen electrode. Actinic light (6000 $\mu E \cdot m^{-1} \cdot S^{-1}$ at the jacket surface) was provided by passing light from a focused tungsten source through a Corning 3-69 filter.

Photochemically active, non-oxygen-evolving Photosystem-II particles were prepared as described [34] with modifications suggested by J. Mullet (personal communication). Following the first Triton solubilization step in [34], nondigested material was removed by centrifugation at $163 \times g$ for 2 min and a pellet obtained from the supernatant by centrifugation at $20\,000 \times g$ for 20 min. This pellet was resuspended to 0.5 mg Chl/ml in 20 mM sodium-tricine (pH 7.8) and 10 mM $MgCl_2$, brought to 0.6% (w/v) in Triton X-100, and incubated for 30 min with stirring at 6°C. The pellet collected by centrifugation at $20\,000 \times g$ for 15 min was resuspended in 20 mM sodium-tricine (pH 7.8) and incubated with digitonin (5 mg/mg Chl) and octylglucopyranoside (1.5 mg/mg Chl) until the suspension appeared bright red under 366 nm illumination (about 5 h). Photosystem-II enriched particles were recovered on linear 0.25–1.5 M sucrose density gradients containing 0.15% (w/v) sodium-cholate and 20 mM sodium-tricine (pH 7.8) by centrifugation in a Ti 60 rotor at 31 000 r.p.m. for 11 h.

Results

Analysis of hcf-3 thylakoids*

We have previously shown that the recessive nuclear *hcf**-3 mutation in maize results in the physical loss of organized Photosystem-II reaction centers [21,28,29]. This loss is accompanied by a reduction in the staining intensity of 32 kDa and 16 kDa lamellar polypeptides, as well as with the loss of the chloroplast-encoded 34–32 kDa polypeptide involved in atrazine binding. Unfortunately, further electrophoretic analysis of *hcf**-3 lamellae was hindered by our inability to resolve polypeptides in the 40–50 kDa region of the gel when examining normal maize thylakoids. Poly-

peptides in this molecular weight range have been implicated as components of the Photosystem-II reaction center by both mutational and fractionation studies.

We show here that the ability to visualize several lamellar polypeptides in maize depends strongly upon the conditions of sample preparation (Fig. 1). When using Laemmli's solubilizing buffer [32], heating samples to 100°C for 3 min resulted in the loss of polypeptides with apparent molecular weights of 110000, 68000, 52000, 48000, 34000 and 14000, a reduction in the staining intensity of a 42 kDa polypeptide, and the appearance of a 22 kDa polypeptide when compared to profiles obtained with samples solubilized on ice (Fig. 1A). These losses were accompanied by the formation of aggregates at the top of the gel. Solubilization of the membranes at 20°C leads to intermediate degrees of loss of the heat labile polypeptides. The heat-sensitive polypeptides all appeared less labile when solubilized in the tricine-based sample buffer used by Metz and Miles [30]. In this buffer only minor changes were seen in the polypeptide profile when thylakoids were solubilized at 20°C, and most of the heat labile polypeptides were still present after heating the sample to 100°C (Fig. 1C and D). Systematic comparison of the two buffers revealed that the pH was the major factor determining stability during solubilization.

The temperature at which gels were run also affected the appearance of the heat-labile proteins, with these proteins disappearing, and aggregation

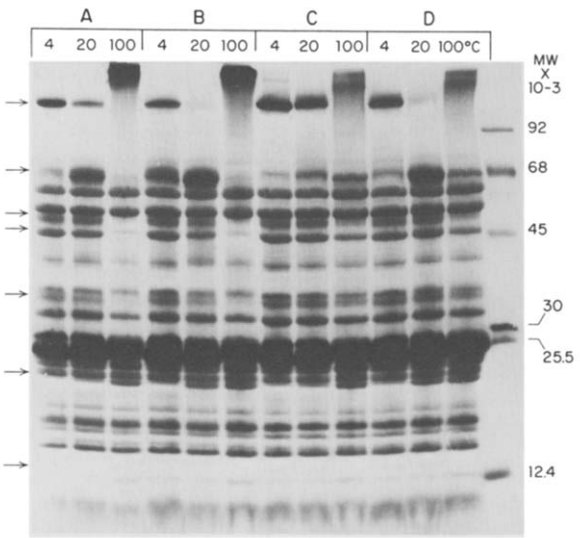

Fig. 1. LDS polyacrylamide gel electrophoresis of wild-type maize thylakoids at 4°C. (A) Thylakoids were solubilized in the sample buffer of Laemmli [32] either on ice (4°C) or at 20°C for 10 min prior to electrophoresis. The 100°C sample was placed in boiling water for 2 min followed by an 8 min incubation at 20°C. (B) Thylakoids solubilized as in (A), but incubated in solubilizing buffer for 45 min prior to electrophoresis. (C) Thylakoids were solubilized in 50 mM sodium-tricine (pH 7.8), 50 mM dithiothreitol, 7% (v/v) glycerol, and 2% (w/v) LDS for 10 min at the indicated temperatures. (D) Thylakoids solubilized as in (C), but incubated in solubilizing buffer for 45 min prior to electrophoresis. All samples were solubilized at a detergent:Chl ratio of 20:1 (w/w). Arrows indicate heat labile proteins.

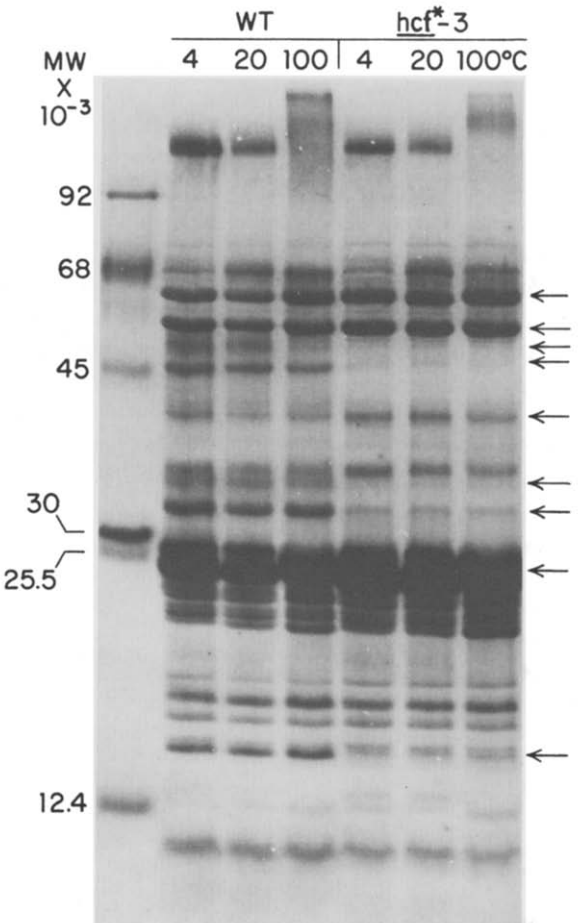

Fig. 2. Lamellar polypeptide profiles obtained from wild-type and *hcf**-3 chloroplasts. Thylakoids were solubilized in sodium-tricine (pH 7.8) based sample buffer for 10 min at the indicated temperatures prior to electrophoresis. Arrows indicate polypeptides present in altered amounts in *hcf**-3 thylakoids when compared to wild type. Chl, 15, μg/lane.

increasing, as the run temperature was raised to 20°C (data not shown). This effect was especially evident when membranes were solubilized in Laemmli's buffer.

A comparison of wild type and *hcf**-3 thylakoids solubilized and run under optimized conditions is shown in Fig. 2. When loaded on an equal Chl basis, *hcf**-3 thylakoids were clearly missing stainable 52, 48 and 34 kDa polypeptides, were deficient in 32 and 16 kDa polypeptides, and were slightly enriched in 59, 54 and 42 kDa and LHCP (25–27 kDa) polypeptides. The major effect of heating either wild-type or *hcf**-3 membranes in tricine (pH 7.8)-solubilizing buffer was the disappearance of the 110 kDa band and the concomitant appearance of protein aggregates at the top of the gel.

Chl a proteins

Several Chl proteins were detected on gels when thylakoids were solubilized under optimal conditions and the duration of the electrophoretic run was minimized (Fig. 3). The nomenclature adopted for naming Chl proteins in this report follows in general that suggested by Machold et al. [3] while retaining CP-1 and LHCP as designations commonly used for these major Chl proteins. As shown in Fig. 3A, all Chl-proteins seen in wild-type profiles were also seen in *hcf**-3, although when loaded on an equal Chl basis the yield of CP-1* and CP-1** was reduced, and that of CPa-2 increased in mutant thylakoids. Since the mobility and Chl absorption spectrum (Fig. 9A) of CPa-2 matched those expected for Chl *a* proteins thought to be associated with the Photosystem-II reaction center, we were surprised to find an enrichment of CPa-2 in *hcf**-3 thylakoids.

In addition to the Chl-proteins visible as green bands, several minor Chl-proteins were detected by their fluorescence (Fig. 3B). Two of these Chl-proteins, CPa-1 and CPa-1*, were present in wild-type membranes but were clearly absent from patterns obtained with *hcf**-3 thylakoids. The absorption spectrum of CPa-1 was similar to that of CPa-2 and indicated a lack of Chl *b* (Fig. 9a). CPa-1 and CPa-1* were highly unstable during long term electrophoresis, while CPa-2 was somewhat more stable. The region of the gel containing these Chl *a* proteins was complex, with additional

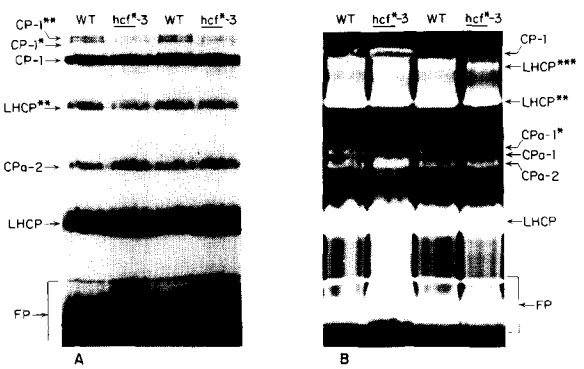

Fig. 3. Chlorophyll proteins visualized in wild-type and *hcf**-3 thylakoids during electrophoresis at 4°C. Lanes were loaded on an equal Chl basis. (A) Green bands. (B) Red bands visualized following activation of Chl fluorescence.

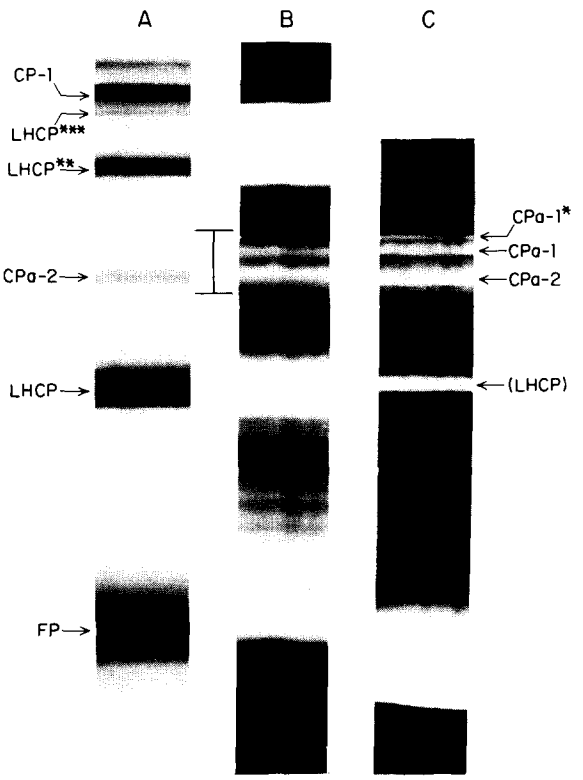

Fig. 4. Preparative electrophoresis of Chl proteins. (A) Green and (B) fluorescent bands visualized during the first cycle of preparative electrophoresis. (C) Fluorescent bands seen following reelectrophoresis of a gel strip excised from the 40–50 kDa region of the first preparative gel as delimited by the bracket. Note appearance of the monomeric form of LHCP* following the second preparative cycle.

minor fluorescent bands sometimes being detected.

Large amounts of these Chl *a* proteins were obtained by preparative electrophoresis. The distribution of Chl proteins during a preparative run was similar to that seen analytically (Fig. 4A and B). The Chl *a*-proteins were further purified by excising a broad (2–4 mm) gel slice in the 'CPa' region and subjecting it to a second cycle of preparative electrophoresis. Care was taken to exclude from the 'CPa' slice any green or fluorescent Chl from the broad LHCP region. All the three Chl *a* proteins were clearly resolved after the second preparative cycle, in addition to a Chl *a/b* protein migrating at the position expected for LHCP (Fig. 4C). It appears that the 'CPa' region contains an oligomer of LHCP, designated LHCP*, which breaks down to 'monomeric' form during the second cycle of purification.

The apoproteins of the minor Chl *a* proteins were characterized by electroelution of protein from gel slices containing twice purified complexes. Slices were not heated prior to electrophoresis as heating resulted in an ability to elute protein; nevertheless, Chl did dissociate from the apoproteins during the analytical run. As shown in Fig. 5A, the apoprotein of CPa-1 comigrated with a 48 kDa lamellar polypeptide. Electroelution of CPa-2 yielded a major 42 kDa polypeptide (partially contaminated by the 48 kDa species), as well as a second polypeptide of about 22 kDa. The 22 kDa polypeptide did not appear after electroelution of CPa-1.

While analysis by electroelution was convenient, we experienced ambiguity in determining molecular weights due to variability in the time taken to elute protein from different gel slices. To circumvent this problem, Chl proteins were extracted from preparative gel strips by homogenization as described in Materials and Methods. Chl was removed from the apoproteins during these procedures. As shown by this analysis, the apoprotein of CPa-1 again comigrated with a 48 kDa lamellar polypeptide. This polypeptide was present in wild type but missing from *hcf**-3 thylakoids (Fig. 5B). Similar analysis of CPa-2

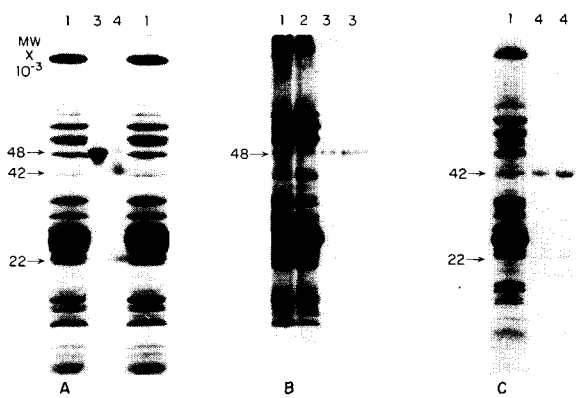

Fig. 5. Analytical electrophoresis of proteins obtained from green or fluorescent gel slices either by electroelution (A) or following extraction of gel slices (B, C). Samples: (1) wild type thylakoids; (2) *hcf**-3 thylakoids; (3) Cpa-1; (4) CPa-2. All Chl-proteins were purified by two cycles of preparative electrophoresis.

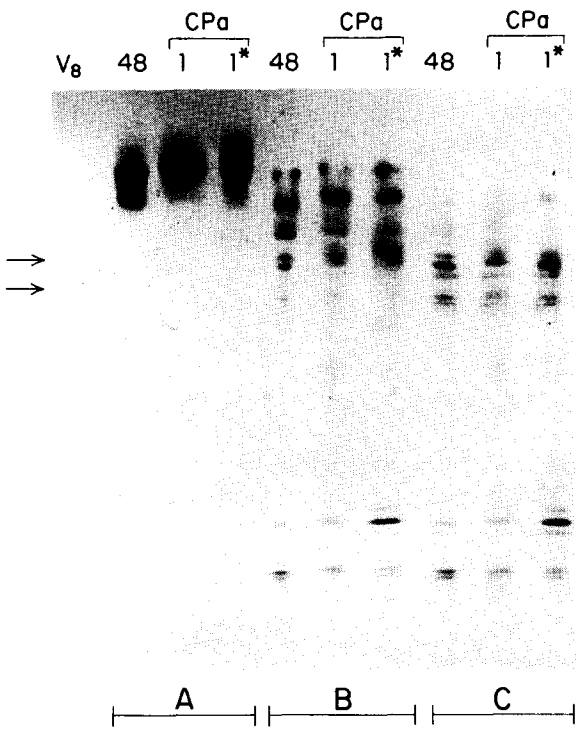

Fig. 6. Partial proteolytic digestion of the CPa-1 and CPa-1* apoproteins and the stained 48 kDa lamellar polypeptide using *S. aureus* V_8 protease. Gel slices containing the stainable 48 kDa polypeptide were obtained from wild type polypeptide profiles. CPa-1 and CPa-1* were purified by one cycle of preparative electrophoresis. Electrophoresis in the absence of protease (A), in the presence of 0.1 μg (B) or 0.5 μg (C) of protease. Arrows indicate autoproteolytic fragments from 0.5 μg of the protease alone.

again revealed major and minor proteins at 42 and 22 kDa, respectively (Fig. 5C).

Because the amount of CPa-1* surviving two cycles of preparative electrophoresis was small, the mobility of the CPa-1* apoprotein was compared with that of the stainable 48 kD lamellar polypeptide and the CPa-1 apoprotein only by electroelution. The major polypeptide eluted from the CPa-1* slice comigrated with the 48 kDa lamellar polypeptide, and was often contaminated by a closely migrating 52 kDa species (Fig. 6).

Proteolytic mapping

To establish further the identity between the 48 kDa polypeptide seen in lamellar polypeptide profiles and the apoproteins of CPa-1 and CPa-1*, the structural relatedness of these polypeptides was compared by partial proteolytic mapping. The cleavage patterns generated using *S. aureus* V_8 protease were strikingly similar for all three polypeptides as determined with two concentrations of the protease (Fig. 6). Most proteolytic comparisons between the CPa-1 apoprotein and the 52 kDa lamellar polypeptide indicated that these two polypeptides were not structurally related (data not shown). This suggests that the 48 kDa lamellar polypeptide is the most likely candidate for the apoprotein of both CPa-1 and CPa-1*.

Photosystem-II particles

The loss of CPa-1, CPa-1*, and the 48 kDa apoprotein from *hcf**-3 suggests that these Chl *a* proteins are closely associated with the Photosystem-II reaction center. The relationship between CPa-2 and the reaction center is less clear, since *hcf**-3 thylakoids contain increased amounts of this Chl *a* protein (Fig. 3). To determine whether CPa-2 is required for Photosystem-II activity, we analyzed the polypeptide composition and chlorophyll-protein content of a highly resolved Photosystem-II particle [34]. The Photosystem-II particle prepared from wild type maize contained major 48, 34, 32 kDa and LHCP polypeptides, and minor proteins at 59, 54 and 52 kDa (Fig. 7A). Only traces of the 110 kDa CP-1 Chl protein were detected. The major Chl protein seen was the LHCP; in the 'CPa' region; a minor green band at the position of CPa-1* was evident (Fig. 7B). Fluorescence visualization revealed the presence of

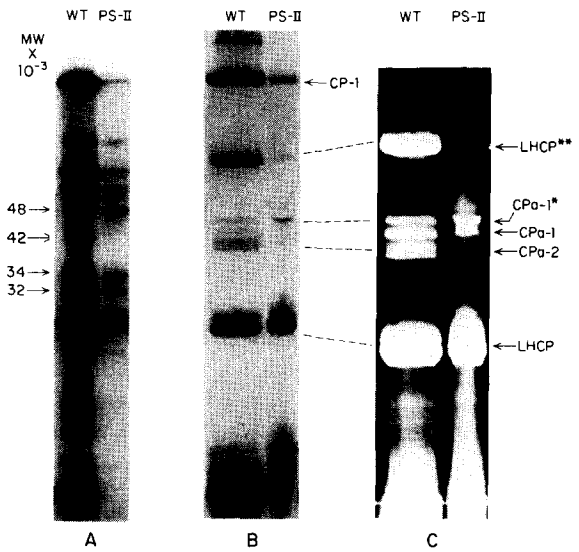

Fig. 7. Polypeptide composition and Chl protein content of detergent-derived PS II particles and unfractionated lamellae. (A) Stainable polypeptide profiles; (B) green; and (C) fluorescent Chl proteins seen in PS-II particles and unfractionated lamellae. Note the loss of CPa-2 from the PS-II particles.

both CPa-1 and CPa-1* in the particles (Fig. 7C), while CPa-2 was not detected. The loss of CPa-2 was not simply due to dissociation of Chl from the apoprotein during particle preparation, since the 42 kDa apoprotein was also missing (Fig. 7A).

The photochemical activity of the maize Photosystem-II particle is shown in Table I. As reported for the original preparation [34] this particle was unable to use water as an electron donor but was able to photoreduce DCIP in a partially DCMU-sensitive manner when provided with diphenylcarbazide. As we could measure only low rates of DCIP reduction in the instrument used for these measurements due to limiting actinic light, an alternative means for measuring Photosystem-II activity was sought. We found that the Photosystem-II specific Mehler reaction catalyzed by DBMIB in the presence of Mn^{2+} [35] could be used to measure electron transport by these particles in either the presence (with a large dark uptake) or absence of diphenylcarbazide. Oxygen uptake by the Photosystem-II particles was again partially DCMU-sensitive, and was completely abolished by heating the particles to 100°C prior to measurement (data not shown). The fact that these Photosystem-II particles were active in the

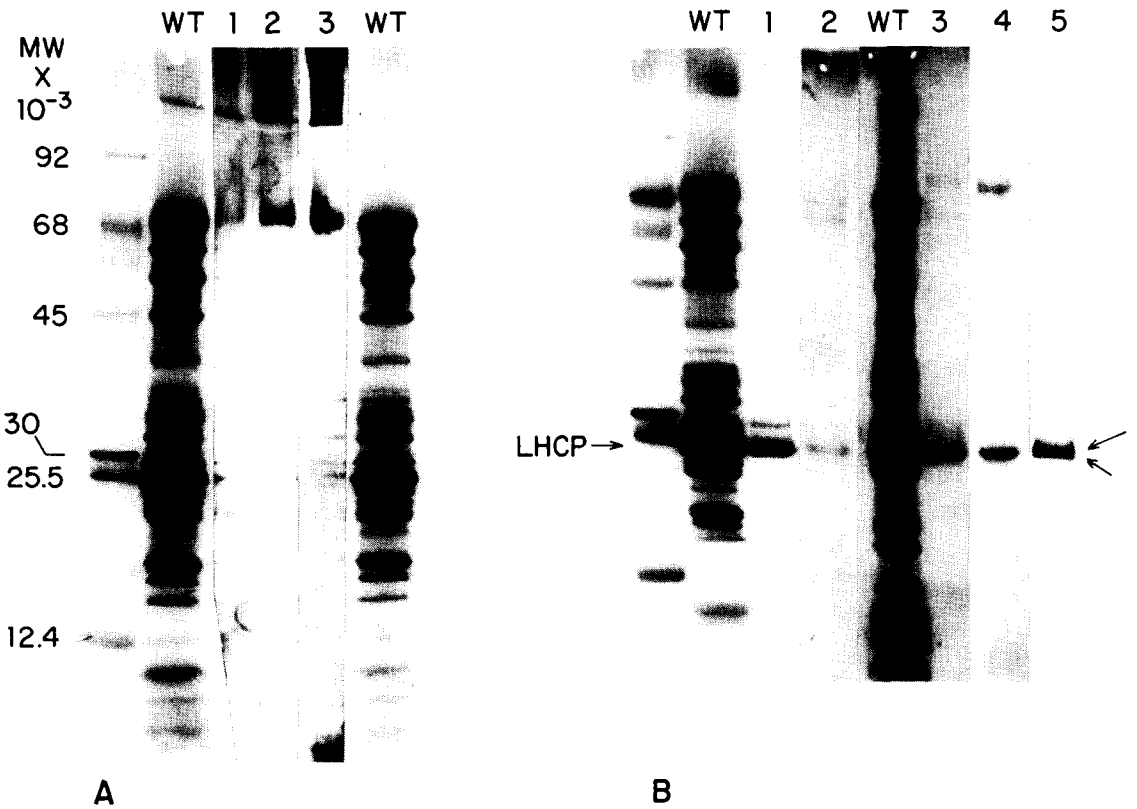

Fig. 8. (A) Stainable polypeptides visualized following electroelution from green gel slices containing CP-1** (1), CP-1* (2) and CP-1 (3). Protein was stained with silver. (B) Analysis of green slices containing the Chl *a*/*b* proteins LHCP (1), LHCP*** (2), LHCP** (3, 4) and the LHCP* monomer (5). Slices were extracted by homogenization following one (2, 3) or two (1, 4, 5) cycles of preparative electrophoresis. Lanes 4 and 5 were silver-stained. A small amount of the LHCP** apoprotein can be seen migrating at its original position in lane 5.

complete absence of CPa-2 indicates that this Chl protein is not obligatorily required for Photosystem-II activity.

Other Chl-proteins

Electrophoretic and spectral analysis of other maize Chl proteins was performed following isolation of the individual complexes on preparative gels. Reelectrophoresis of CP-1, CP-1* and CP-1** resulted in the appearance of polypeptides of 110 and 68 kDa (Fig. 8A). All three of these Chl-proteins contained Chl *a* as evidenced by a prominent absorption maximum near 670 nm and little or no Chl *b* as determined by the absence of a shoulder at 652 nm (Fig. 9A). These characteristics are consistent with those previously reported for CP-1 and its oligomers [3,4,5,10].

Reelectrophoresis of extracts obtained from gel slices containing LHCP***, LHCP**, and the 'monomeric' form of LHCP* yielded polypeptides comigrating with 'monomeric' LHCP in each case (Fig. 8B). When loaded lightly, we could resolve the pigmented monomer into a green or fluorescent doublet [36–39]; such a stained doublet is evident in lane 5. LHCP** was also occasionally resolved as a fluorescent doublet (data not shown). All three oligomers and the monomer contained both Chl *b* (absorption maxima at 652 and 648 nm) and Chl *a*. Thus, many of the Chl proteins seen in the maize profile appear to be oligomers or higher order complexes of CP-1 and LHCP.

Discussion

By employing conditions designed to minimize the loss of heat labile polypeptides, we were able

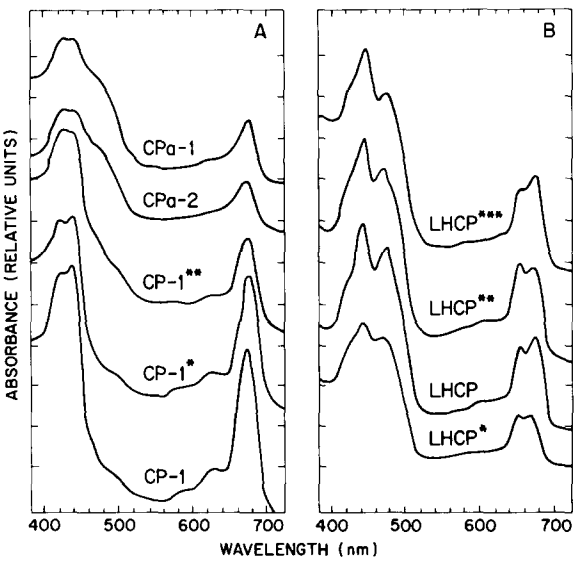

Fig. 9. Absorption spectra of Chl proteins scanned in gel slices. CPa-1, LHCP**, LHCP, and LHCP* monomer were recovered following two cycles of preparative electrophoresis. Other Chl proteins were purified by a single cycle. (A) Chl *a* proteins. (B) Chl *a*/*b* proteins.

TABLE I

ELECTRON-TRANSPORT ACTIVITIES OF WILD TYPE CHLOROPLASTS AND PHOTOSYSTEM-II PARTICLES

Electron-transport assays were performed in a reaction mix containing 10 mM sodium-tricine (pH 7.8), 10 mM NaCl, 0.1 M sorbitol, and 10 μg Chl, with the addition of 10 μM DCIP, 20 μM DBMIB, 100 μM Mn^{2+}, 0.5 mM DCBQ, 1 mM FeCN, 1–2 mM diphenylcarbazide, and DCMU as indicated. Final reaction volumes were 3 ml for DCIP reduction and 1 ml for measurements of oxygen volution (+) and consumption (−). Rates: μM O_2 evolved/consumed, or μM DCIP reduced·mg $Chl^{-1}\cdot h^{-1}$.

Reaction	Rate	
	Wild-type	PS-II particles
$H_2O \rightarrow$ DCIP	134	0
diphenylcarbazide → DCIP	–	66
diphenylcarbazide → DCIP +10 μM DCMU	–	14
$H_2O \rightarrow$ DCBQ/FeCN	(+)238	0
diphenylcarbazide → DBMIB/Mn^{2+}	–	(−)180
$H_2O \rightarrow$ DBMIB/Mn^{2+}	(−)150	(−)181
5 μM DCMU	(−)25	–
10 μM DCMU	–	(−)107
100 μM DCMU	–	(−)83

to resolve 10 Chl proteins in wild type miaze thylakoids (Fig. 1). Two of these Chl proteins, CPa-1 and CPa-2, lacked Chl *b* and were found to contain major apoproteins of 48 and 42 kDa, respectively (Fig. 5). These characteristics are typical of Chl *a* proteins thought to be associated with the Photosystem-II reaction center [3,4,7,13,20, 22–24]. The data presented in this report indicate that of the two Chl *a*-proteins, CPa-1 is more closely associated with the Photosystem-II reaction center than is CPa-2. Two lines of evidence support this contention. First, *hcf**-3 thylakoids lacking Photosystem-II reaction centers exhibited the complete loss of CPa-1 and its 48 kDa apoprotein while retaining elevated levels of both CPa-2 and its major 42 kDa apoprotein (Figs. 2 and 3). This establishes a correlation between the loss of Photosystem-II reaction centers and the specific loss of CPa-1. Second, the photochemically active Photosystem-II particles prepared in this study (Fig. 7, Table I) contained CPa-1 but exhibited the complete loss of CPa-2 and its major 42 kDa apoprotein. This indicates that CPa-2 is not necessary for Photosystem-II activity, and establishes CPa-1 as the species most likely to contain the photoactive chlorophyll of Photosystem II.

Camm and Green have described the enrichment of two Chl *a* proteins in Photosystem-II particles prepared by extraction of thylakoids with octylglucoside [7,13,24]. These Chl-proteins, most recently designated CPa-1 (47 kDa) and CPa-2 (43 kDa), appear similar to CPa-1 and CPa-2 described in this report. Recently, a partial separation of CPa-1 and CPa-2 was achieved following fractionation of the extract on sucrose density gradients [25]; the distribution of Photosystem-II activity followed primarily that of CPa-1 across the gradient. While it was not determined whether the distribution of the Chl *a*-proteins was a true reflection of the distribution of their constituent apoproteins, these data, like ours, strongly support the contention that CPa-1 contains the photoactive Chl of Photosystem II.

In contrast to the situation in algae [22,23], the loss of CPa-1 from *hcf**-3 thylakoids was not accompanied by the concomitant loss of CPa-2. This demonstrates that the mutational loss of these Chl *a* proteins is not necessarily coordinate, and may explain the persistence of minor Chl *a* pro-

teins in certain Photosystem-II-deficient maize and barley mutants [3,19]. This differential loss may be due to differences in the rates of synthesis and/or turnover of the apoproteins in mutant thylakoids, or may reflect different functional roles for the two Chl *a* proteins. We have recently found a preferential enrichment of CPa-2 in the light (D-144) fraction following disruption of thylakoids with digitonin (data not shown); this observation, as well as the association of a 22 kDa polypeptide with CPa-2 (Fig. 5), requires further investigation.

While CPa-1 seems most likely to contain the active chlorophyll of Photosystem II, Machold et al. [3] suggested previously that a Chl *a* protein similar to CPa-2 was sufficient to support Photosystem II activity in the absence of CPa-1 [3]. This conclusion was based on the observation that a Photosystem-II-deficient, but nevertheless autotrophic barley mutant, viridis-m^{29}, lacked a Chl *a*-protein designated Chl *a* P2 while retaining a second Chl *a* protein, Chl *a* P3. These Chl *a* proteins appear to resemble CPa-1 and CPa-2, respectively. We disagree with this interpretation based on our characterization of the Chl-protein content of Photosystem-II particles and the marked instability of CPa-1, and suggest that the failure to detect Chl *a*-P2 in viridis-m^{29} was a consequence of the reduced content of Photosystem-II reaction centers in mutant thylakoids.

In addition to CPa-1 and CPa-2, we have identified a third minor Chl-protein which migrates slightly behind CPa-1 during electrophoresis. Like CPa-1, this Chl-protein appears to be an integral part of the Photosystem II reaction center, since it was lost from *hcf**-3 thylakoids and was enriched in purified Photosystem-II particles (Figs. 3 and 7). Proteolytic mapping indicated that the apoprotein of this complex and the 48 kDa CPa-1 apoprotein were structurally similar (Fig. 6). We feel that this Chl protein is most probably an electrophoretic variant of CPa-1 and have designated it CPa-1*. We recognize that this analysis is complicated by the presence of a diffuse, closely migrating 52 kDa polypeptide which also appears to be associated with Photosystem II [30]. However, repeated proteolytic comparison between CPa-1 and the stainable 52 kDa polypeptide indicated that these polypeptides were structurally dissimilar (data not shown). The low content of this Chl protein on gels precluded a spectral determinant of pigment content.

The minor Chl *a* proteins described in this report appear to be similar to previously described Chal *a* proteins on the basis of relative mobility and the apparent molecular weights of their apoproteins. By these criteria, CPa-1 and CPa-2 resemble, respectively, complexes III and IV in *Chlamydomonas* [20,22,23], Chla-P2 and Chl *a*-P3 in barley [3], and CPa-1 and CPa-2 in soybean cotyledons [4]. The A-2 complex described in Miles et al. [19] probably resembles CPa-2.

In addition to the minor Chl *a*-proteins discussed above, several other Chl proteins were resolved during the course of this study and were identified as being oligomers or higher-order complexes of LHCP or CP-I (Fig. 8). While most of these complexes have been described by others [3–10], the resolution of a higher molecular-weight variant of LHCP migrating in the 'CPa' region (designated here as LHCP*, (Fig.4)) has been described only once previously [3]. Since most of these complexes were present, to varying degrees, in Chl protein profiles from *hcf**-3 thylakoids (Fig. 3), it appears that these thylakoids are not so severely disrupted as to prevent the association of Chl proteins into higher order complexes.

Since the preparation of this manuscript, H.Y. Nakatani has reported that an electrophoretically isolated Chl *a* protein, CP47, exhibits a 695 nm low temperature fluorescence emission peak and other optical signals thought to arise from P-680 and pheophytin associated with the Photosystem II reaction center [40]. Based on electrophoretic mobility, CP 47 most probably corresponds to CPa-1 described in this report.

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