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Purification and characterization of an iron stress-induced chlorophyll-protein from the cyanobacterium *Anacystis nidulans* R2

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An *Anacystis nidulans* R2 chlorophyll-protein associated with Photosystem II in iron-stressed cells (Pakrasi, H.B., Riethmann, H.C. and Sherman, L.A. (1985) Proc. Natl. Acad. Sci. USA 82, 6903–6907) has been biochemically purified and characterized. Anion exchange chromatography of dodecyl-β-D-maltoside-solubilized membranes from iron-deficient cells was used to recover this chlorophyll-protein (termed CPVI-4) in high yield and in a relatively native state. CPVI-4 has a room temperature absorption maximum at 671 nm, a 77 K chlorophyll fluorescence peak at 681 nm, and contains polypeptides of 36, 34 and 12 kDa. The 36 and 34 kDa polypeptides are associated with chlorophyll on mildly denaturing acrylamide gels of purified CPVI-4, although only the 34 kDa protein is immunoreactive with antisera elicited against the gel-purified chlorophyll-protein. Immunoblotting experiments with dodecyl-β-D-maltoside-solubilized membrane fractions and purified CPVI-4 indicate that CPVI-4 does not contain previously identified Photosystem II core proteins. CPVI-4 likely functions as a light-harvesting antenna complex in iron-starved cells (where phycobilisomes are absent or diminished) and, in addition, may contribute chlorophyll to the reaction center complexes during their assembly in the early stages of recovery from iron stress.

Introduction

Anacystis nidulans R2 cells grown in the absence of exogenously added iron develop a very distinctive phenotype [1,2] with substantially altered spectroscopic properties. The number of thylakoid membranes per cell decreases to 25% or 30% that of normal cells [3], high molecular mass chlorophyll-protein (CP) complexes typical of normally grown cells are highly depleted [4,5], and the ratio of phycocyanin to chlorophyll is decreased substantially [1,4,5]. The long-wavelegth 77 K Chl fluorescence emission peaks (716 nm and 696 nm) decrease relative to the 685 nm peak, and the normal room temperature Chl absorption maximum at 680 nm is blue-shifted to 672 nm in iron-stressed cells [1,6]. Concurrent with these changes is the accumulation of a novel CP complex, termed CPVI-4 [7]. When illuminated with 435 nm light at 77 K, gel-isolated CPVI-4 emits fluorescence with a sharp peak at 685 nm, typical of Chl associated with PS II [7]. Furthermore, CPVI-4 was found to copurify with PS II particles

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Abbreviations: Chl, chlorophyll; CP, chlorophyll-protein complex; DβD-maltoside, dodecyl-β-D-maltoside; F_{max}, maximal fluorescence; F_0, original fluorescence; F_v, variable fluorescence; LHC, light-harvesting chlorophyll-protein; PS II, Photosystem II; PS I, Photosystem I.

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from *Anacystis nidulans* R2 [8]. These results suggested an association of CPVI-4 with cyanobacterial PS II, either as a light-harvesting complex or as a part of the reaction center core [7].

The Chl in thylakoids from normally grown *Anacystis nidulans* is associated with six major Chl-protein bands on polyacrylamide gels (reviewed in Ref. 2). CPI, CPII, CPIII, and CPIV are each high molecular weight CP complexes containing the PS I Chl-protein and its associated 'core' subunits. Additional polypeptides of unknown function are also found in these complexes; among these are proteins of 36 and 35 kDa which are prominent in CPIII and CPIV [9]. CPV is a very minor band that probably represents a monomeric form of the PS I reaction center [9]. CPVI contains PS II-associated Chl-proteins, and has been resolved into four distinct Chl-containing bands [7]. CPVI-1 is associated with the phycobilisome anchor polypeptide as well as an unidentified 42 kDa protein, CPVI-2 and CPVI-3 are cyanobacterial analogs of plant chloroplast CP47 and CP43, respectively, and CPVI-4 contains a 36 kDa protein [7]. CPVI-4 can barely be detected in normally grown *A. nidulans* cells, but accumulates to very high levels in iron-starved cells [7].

Analysis of *A. nidulans* Chl-protein composition upon iron stress and during recovery from iron stress indicates that CPVI-4 is associated with most of the Chl in iron-starved cells [7,8]. PS II core polypeptides (including CP47, CP43, D1, and D2) can barely be detected, and the high molecular weight Chl-proteins, CPI, CPII, CPIII, and CPIV (which contain most of the Chl in normal cells) are also highly depleted in iron-starved cells [2]. PS I is present in low levels in iron-starved cells, and migrates primarily in CPIII [3,5,8]. Upon addition of iron, the levels of each of the Chl-proteins increase, except CPVI-4, which decreases [8]. Because of the abundance of CPVI-4 in iron-starved cells and the distribution of Chl among CP bands during recovery from iron stress, it seems likely that CPVI-4 plays a key role in the development of the *A. nidulans* thylakoid upon recovery from iron stress.

In order to characterize in more detail the structure of this Chl-protein, its interaction with thylakoid components, and its regulation by iron stress, it was necessary to devise a simple protocol for the preparation of large amounts of purified CPVI-4. We describe such a procedure in this paper, along with a biochemical and spectral characterization of purified CPVI-4.

Materials and Methods

Growth conditions and membrane preparation. The characteristics and growth conditions of *Anacystis nidulans* R2 grown in either nutrient-sufficient or iron-limiting media have been reported previously [3]. Frozen cells collected from iron-starved cultures were thawed and broken by passage through a chilled French pressure cell, and membranes were isolated from the broken cells using differential centrifugation as described previously [7]. *Anacystis nidulans* R2 membrane components were separated into hydrophobic and hydrophilic fractions using the Triton-X114 phase partitioning system of Bordier [10] as modified by Bricker and Sherman [11].

Purification of CPVI-4. Membranes (containing 9 mg Chl) were isolated from iron-stressed cells and were suspended to 0.5 mg Chl/ml in 25 mM Tris (pH 8.2)/10 mM NaCl. Dβ-D-maltoside was added to 2% (wt./vol.), and the sample was incubated on ice for 30 min. After centrifugation (150000 × *g*, 1 h, Beckman 70 Ti rotor) to remove insoluble material, the extract (containing 5.6 mg Chl) was loaded onto a DEAE Sephacel column (Sigma Chemical Co., St. Louis, MO); 15 ml of extract was loaded onto a 2.5 cm × 35 cm column equilibrated in a solution comprised of 25 mM Tris (pH 8.2)/10 mM NaCl/0.05% (wt/vol) Dβ-D-maltoside. A yellow fraction eluted while loading, but all green material was retained on the column. The column was developed using a 10–300 mM NaCl linear gradient in the equilibration buffer, and the eluate was collected in 7 ml fractions. The peak of the CPVI-4-containing fractions were pooled, diluted with four volumes of equilibration buffer, and loaded onto a small (1.5 cm × 6 cm) DEAE Sephacel column equilibrated in the same buffer as the first column. After washing the loaded column with equilibration buffer, the small column was developed with a 10–200 mM NaCl linear gradient in equilibration buffer, and eluant was collected in 1.4 ml fractions. Peak Chl-containing fractions were analyzed

as to spectral characteristics and polypeptide composition. Acquisition of absorption and fluorescence spectra was performed as described in Ref. 12.

Electrophoresis and immunoblotting. Electrophoresis conditions for the analysis of both intact Chl-proteins and denatured polypeptides have been described previously [7,13]. Polypeptides were visualized in gels by Coomassie blue staining (Bio-Rad) or by the silver staining method of Wray et al. [14]. Immunoblots of gels were prepared as described previously [15,16].

Antibody preparation and sources. Antisera were generated against the proteins associated with CPVI-4 after denaturing electrophoresis of excised green gel bands. The major protein band from preparative gels of CPVI-4 material was electro-eluted, emulsified in Freund's incomplete adjuvant, and injected subcutaneously into New Zealand White Rabbits. Approx. 150 μg of protein was included in the initial immunization, and booster injections containing 75 μg of protein were given as 3 and 5 weeks. Rabbits were bled and killed at 7 weeks, and the IgG fraction of sera was collected by precipitation with ammonium sulfate. The purified IgG was dissolved in a small volume of buffer containing 1% (wt./vol.) bovine serum albumin/0.1% (wt./vol.) NaN_3, and stored frozen at −70°C in small aliquots. A 10000:1 dilution of this stock was used for immunodecoration of protein blots.

Antibodies to *Chlamydomonas reinhardtii* thylakoid proteins p5 and p6 were provided by Dr. N-H Chua (Rockefeller University, New York) [18,19]. Antisera to spinach chloroplast proteins D1 and D2 were a gift of Dr. Y. Inoue (RIKEN, Japan) [20].

Results

Biochemical purification of CPVI-4

The nonionic detergent Dβ-maltoside was found to effectively solubilize *A. nidulans* R2 membrane material from both normal and iron-deficient cells, and most of the solubilized Chl remained stably bound in Chl-protein complexes even after mildly denaturing electrophoresis resolved these Chl protein complexes [7]. Because of these unique properties, Dβ-maltoside was chosen as the solubilizing agent for the isolation of CPVI-4 from membrane material obtained from iron-stressed cells. Preparative quantities of solubilized material were chromatographed on a DEAE Sephacel column as described in Materials and Methods and the Chl elution profile of this material is shown in Fig. 1 (top). Analysis of Chl-proteins present in the separate fractions as well as immunoblotting procedures using monospecific antibodies to identified PS II proteins [7,18,20] showed that the main Chl peak contains primarily CPVI-4, the left shoulder (fraction 37) is due to CPVI-3 (CP43 analog) and CPVI-4, and

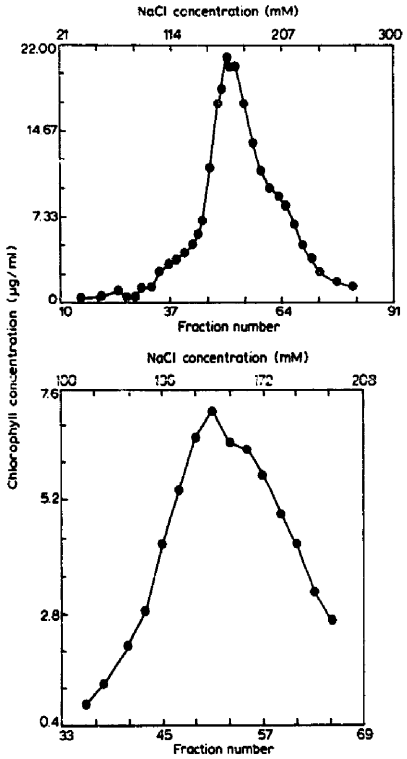

Fig. 1. Fractionation of maltoside-solubilized membranes from iron-stressed cells on DEAE-Sephacel. Top: Chl concentration of column fractions from the first DEAE-Sephacel column. Bottom: Peak CPVI-4 fractions 46–51 from the first DEAE column were pooled, diluted in column buffer, and applied to the small DEAE-Sephacel column. The Chl elution profile is shown.

the right shoulder is due to CPVI-2 (CP47 analog) and CPVI-4. The two proteins now thought to constitute the PS II reaction center, D1 and D2, eluted in the same fractions as CPVI-2 (peaking at fractions 64–70). PS I eluted at a much higher ionic strength (about 0.5 M NaCl), and contained some CPVI-4 material along with the 60 kDa, Chl-binding PS I subunit (data not shown). Chromatography of the solubilized material thus allowed resolution of most of CPVI-4 from the other Chl-proteins present in membranes from iron-stressed cells, and indicated that D1, D2, and 47 kDa co-eluted from the column in fractions depleted of the 43 kDa protein.

Fractions 46–51 from the first column contained the most highly purified CPVI-4, so this material was pooled and re-chromatographed on a smaller DEAE-Sephacel column. The Chl elution profile of this material is shown in Fig. 1 (bottom). The column fractions were analyzed as before, and this analysis indicated that all detectable CPVI-3 and CPVI-2 which had remained in the pooled fractions from the first column were removed by the second chromatography step. Interestingly, double peaks were observed in the elution profiles of both columns, and were centered in the fractions containing the most CPVI-4. Chl-protein analysis using green gels showed no obvious differences in these fractions (data not shown). The possible origin of the double peak is discussed further below.

Spectral characterization of purified CPVI-4

The pigment organization of membranes from iron-stressed cells is very different from that found in normally grown cells. Particularly conspicuous are a shift of the absorbance spectrum from 680 nm to 672 nm, and the presence of a single 685 nm Chl fluorescence emission peak at 77 K, instead of the three peaks at 685 nm, 695 nm and 716 nm seen in normal cells. CPVI-4 is very abundant in iron-stressed cells, whereas phycobilisomes, CPVI-2, and CPVI-3 are much diminished [7,8].

The possible contribution of CPVI-4-associated Chl to these iron-stress phenotypes was assessed in Figs. 2 and 3. Dβd-maltoside extracts of membranes from iron-stressed cells contain all of the known Chl-proteins of these membranes [7], and

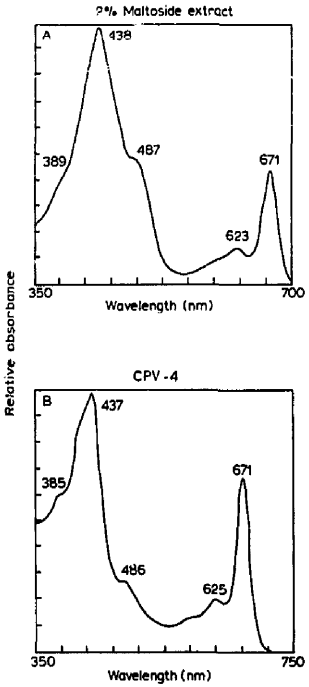

Fig. 2. Absorption spectra of maltoside-solubilized membrane material and purified CPVI-4. Spectra were obtained at room temperature in the column buffer solution.

exhibit major absorption and Chl fluorescence properties typical of iron-stressed cells. Fig. 2 shows that the main Chl absorption peaks are the same in purified CPVI-4 as in the maltoside extracts (671 nm and 437 nm), and that the only differences in these spectra can be attributed to the differential content of carotenoid. This is consistent with the overwhelming abundance of CPVI-4 in green gels of solubilized membranes from iron-stressed cells [7,8]. The absorption peaks originating from the small amount of Chl associated with PS I, CPVI-2 and CPVI-3 in iron-stressed membranes is presumably masked by the much larger absorbance of the Chl *a* molecules associated with CPVI-4. By contrast, purified CPVI-4 has a different carotenoid content than the total maltoside extract; this suggests that a large amount of carotenoid is bound to species other than CPVI-4 in these membranes. A major carotenoid component of *A. nidulans* has been identi-

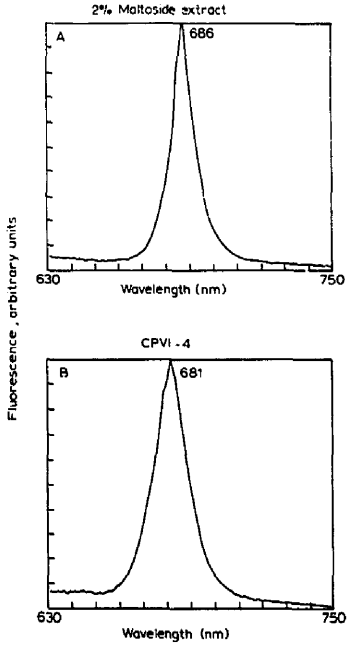

Fig. 3. Low temperature (77 K) Chl fluorescence emission spectra of maltoside-solubilized membrane material and purified CPVI-4. Samples were frozen in the column buffer (at a concentration of about 10 μg Chl per ml).

fied as a cell envelope (either cytoplasmic membrane or outer membrane-associated) carotenoid-binding protein [21,22], and its relative accumulation increases slightly in iron-stressed cells [8]. Part of the depletion of carotenoid in CPVI-4 relative to the maltoside extract is due to the removal of this envelope carotenoprotein from the purified material (data not shown).

The 77 K Chl fluorescence properties of the maltoside extract from iron-stressed membranes and purified CPVI-4 are shown in Fig. 3. The 77 K Chl fluorescence emission spectrum of maltoside extracts (Fig. 3A) is virtually identical to that found in intact iron-starved cells, in isolated membranes, and in gel-purified CPVI-4 [7]. The emission peak of biochemically purified CPVI-4 is shifted to 681 nm (Fig. 3B); this shift is probably due to some disruption of the native Chl *a* organization during anion-exchange chromatography. The 681 nm peak is fairly typical of biochemically purified light-harvesting Chl-proteins [23,24]. As maltoside is present in both the purified CPVI-4 fraction and in the crude extract, the shift in the fluorescence peak is probably not specifically due to this detergent.

Polypeptide analysis of CPVI-4

The polypeptide composition of samples representing major purification steps are shown in Fig. 4. Most of the protein components of the maltoside extract (lanes 2 and 3) are removed after fractionation on the first column (lanes 4 and 5), and the only visible bands after the second column are those migrating at 36, 34, and 12 kDa (minor contaminants in the purified fraction be-

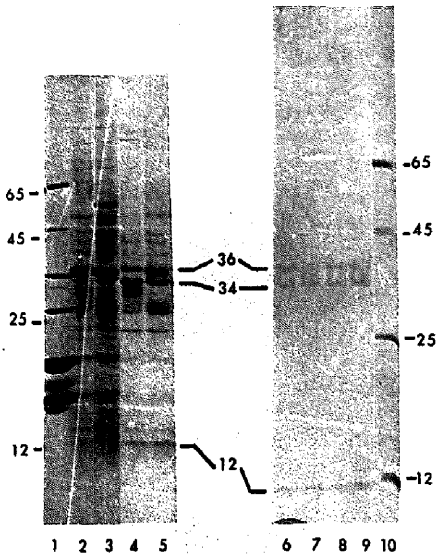

Fig. 4. Polypeptide composition of purified CPVI-4. Maltoside-solubilized material from iron-deficient membranes (lanes 2 and 3) and peak CPVI-4 fractions from the first (lanes 4 and 5) or the second (lanes 6-9) DEAE-Sephacel columns were subjected to lithium dodecyl sulfate-polyacrylamide gel electrophoresis, and the separated components were stained with Coomassie blue. Lane 1 contains purified *A. nidulans* R2 phycobilisomes, and lane 10 contains mol. wt. standard proteins bovine serum albumin (65 kDa), ovalbumin (45 kDa), chymotrypsinogen A (25 kDa), and cytochrome *c* (12 kDa). Lanes 2, 4, 6, and 8 were solubilized at 0 °C, and Lanes 1, 3, 5, 7, 9 and 10 were solubilized at 70 °C for 10 min. The gel on the right side of the figure was run longer than normal to resolve the 30 to 40 kDa region better.

come apparent upon staining gels of purified fractions with silver; see Fig. 6). The CPVI-4 proteins are easily detected in the maltoside extract (lanes 2 and 3) and represent a significant fraction of the total protein associated with iron-stressed membranes [8]. This is consistent with CPVI-4's dominance of spectral properties in iron-stressed cells as described above. Interestingly, the 36 kDa component of CPVI-4 becomes a fuzzy band upon solubilization at 70 °C (lanes 7 and 9), a property shared with the Chl-binding subunit of PS I as well as with some other integral membrane proteins. The polypeptide analysis indicates that CPVI-4 represents a major protein fraction of membranes from iron-stressed cells, and consists of only 3 major polypeptide subunits.

Biochemically purified CPVI-4 fractions were resolved on mildly denaturing lithium dodecyl sulfate-polyacrylamide gels in the presence of maltoside [7] to assess which subunits might bind the Chl *a* associated with the complex. As described previously, these electrophoresis conditions preserve the integrity of the Chl-protein interaction such that most Chl molecules remain bound to their respective apoprotein. Purified CPVI-4 samples were brought to 0.20% (wt./vol.) maltoside, and were loaded directly onto a 5–15% acrylamide gradient gel. Fig. 5 is representative of the results found with all purified CPVI-4 fractions depleted of other Chl-proteins. A single, major green band was present, as well as a series of minor bands (termed a, b and c). All bands were highly fluorescent, and some free pigment dissociated from the Chl-protein during electrophoresis.

Electrophoretic analysis of denatured proteins present in each of these green bands is shown in Fig. 6. Lanes 3 and 4, and lanes 10 and 14 contain the major green band from the peak CPVI-4 fractions of two separate experiments. The total polypeptide composition of peak CPVI-4 fractions from the first DEAE column (a separate experiment from that shown in Fig. 4, lanes 4 and 5) is shown in lanes 1 and 2 for comparison, and the gel was stained with silver. Immediately apparent are the dominance of the 36 kDa and 34 kDa proteins in each of the denatured green bands, and the absence of the 12 kDa protein in all denatured green bands. This suggests that the 12

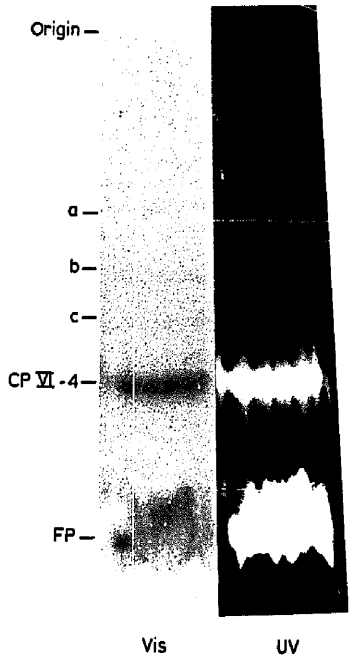

Fig. 5. Green gel of purified CPVI-4. Electrophoresis was carried out under mildly denaturing conditions on a 5–15% gradient acrylamide gel, and green or fluorescent Chl-containing bands were photographed as described in Materials and Methods.

kDa protein is not directly involved in Chl *a* binding, but instead might serve an ancillary function in the complex. Closer inspection of the protein patterns shows a differential quantity of the 36 and 34 kDa subunits, depending on which green band was analyzed. In the case of the major green band (Fig. 5, CPVI-4), the 36 and 34 kDa polypeptides were present in approximately equal amounts (See Fig. 6, lanes 3 and 4). If this band was solubilized at 0 °C, a dense region of staining encompassed both bands (lanes 3 and 10); when the gel was stained with Coomassie, this region appeared as two discrete bands at 36 and 34 kDa. If this material was incubated in sample buffer at 70 °C for 5 min, the 36 kDa band became very diffuse, forming a smear in the 27–30 kDa range as well as in the 34–36 kDa range (lanes 4 and 14). When gels containing the heated samples were stained with Coomassie, the 36 kDa band

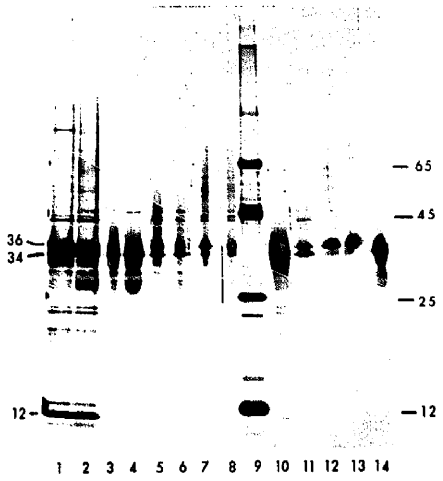

Fig. 6. The protein composition of green bands from purified CPVI-4 fractions. The green bands described in Fig. 5 were excised from the gel, incubated in sample buffer for 10 min at 0 or 70°C, then placed in wells of a 10–20% acrylamide gradient gel with a spatula and overlaid with the same sample buffer in which the gel piece was incubated. After electrophoresis, the gel was stained with silver. Lane 9 contains the same mol. wt. standards described in Fig. 4. Lanes 1 and 2: pooled fractions 46–51 from the first DEAE column, loaded directly onto the denaturing gel; lanes 3 and 4: CPVI-4 derived from a green gel of fraction 47 from the first DEAE column; lanes 5 and 6: CPVI-4 "b" from the same green gel lane; lanes 7 and 8: CPVI-4 "a" from same green gel lane; lanes 10 and 14: CPVI-4 from a green gel of pooled fractions 46–51 from the first DEAE column (material in lanes 10 and 14 and in lanes 3 and 4 were from separate CPVI-4 purification experiments); lanes 11–13: CPVI-4 "c", "b", and "a", respectively, from a green gel of pooled fractions 46–51 from the first DEAE column. Lanes 2, 4, 6, 8, 9 and 14 were heated at 70°C prior to loading, whereas the other samples were held on ice.

stained very poorly, whereas the 34 kDa protein remained as a discrete band of the same staining intensity (data not shown; see Fig. 4, lanes 6–9 for comparison).

The higher mol. wt. green bands a, b and c from Fig. 5 contained much less Chl, and, as is apparent in Fig. 6 (lanes 5–8 and 11–13), much less protein than that found in CPVI-4. Variable amounts of 42 and 46 kDa contaminants could sometimes be found in these bands, as could variable ratios of the 34 kDa and 36 kDa proteins. The two higher mol. wt. bands (Fig. 5, 'a' and 'b') were particularly enriched in the 36 kDa species, although trace amounts of the 34 kDa protein could still be detected. Since only small amounts of Chl were associated with these two bands, we cannot conclude from this that the 36 kDa polypeptide is capable of binding Chl by itself. Conversely, no green band was resolved which contained only the 34 kDa protein. The high molecular mass green bands may in fact be detergent-induced aggregates of partial CPVI-4 complexes. Thus, we conclude from these data that the Chl-binding components of CPVI-4 are polypeptides migrating at 36 kDa and 34 kDa; these two proteins associate with Chl *a*, carotenoid, and a 12 kDa protein to form the iron-stress-induced membrane complex CPVI-4.

Immunoblot analysis of CPVI-4

We had previously generated antisera against CPVI-4 material prepared by excision of the green band from mildly denaturing gels, solubilization of this material at 70°C followed by denaturing gel electrophoresis, and then electroelution of the major Coomassie-stained band [17]. These antisera were reactive with several iron-regulated proteins [8,17] as shown in Fig. 7. The antibody reacts with polypeptides migrating at 36, 35 and 34 kDa in membrane samples from iron-stressed cells (lane 1). An immunoblot analysis of purified CPVI-4 using the same anti-CPVI-4 antibody shows that the antibody reacts only with the 34 kDa component of purified CPVI-4, whether the material was solubilized on ice (lanes 2 and 3) or at 70°C (lane 4) prior to electrophoresis. The 36 kDa protein of CPVI-4 was not detected using this antibody, indicating significant structural differences between the 36 and 34 kDa components of CPVI-4. The immunoreactive 36 and 35 kDa proteins present in iron-starved cells are separate, iron-regulated polypeptides not present in biochemically purified CPVI-4; thus, they were contaminants in the gel-purified CPVI-4 fraction originally used to generate the antisera.

In separate experiments, neither the 36 kDa nor the 34 kDa proteins of purified CPVI-4 were found to be immunoreactive with antisera to the D1 or D2 polypeptides from spinach chloroplasts (data not shown). Antibodies to *Chlamydomonas reinhardtii* chloroplast proteins p5 and p6 also

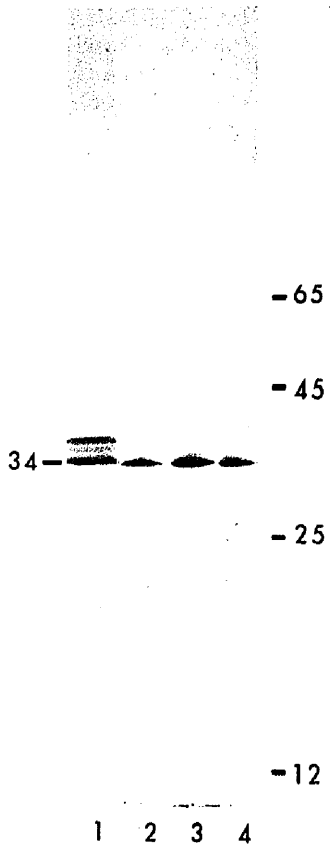

Fig. 7. Reactivity of p34 with antisera raised against electro-eluted CPVI-4. A gel similar to that in Fig. 6 was blotted onto a nitrocellulose filter and probed with antisera to CPVI-4. Lane 1: membranes from iron-deficient cells; lanes 2–4; CPVI-4 prepared from green gels of purified material eluting in the peak, Chl-containing DEAE Sephacel column fractions, incubated in electrophoresis sample buffer at 0 °C (lanes 2 and 3) or 70 °C (lane 4) prior to denaturing electrophoresis.

lacked immunoreactivity with purified CPVI-4. Since all four of these antibodies did recognize the appropriate *Anacystis nidulans* R2 membrane components from iron-stressed cells, a role for the 36 and 34 kDa proteins in PS II reaction center function is unlikely.

Antibodies to LHC II proteins from maize also failed to show immunoreactivity with the CPVI-4 apoproteins, and the anti-CPVI-4 antibodies reactive with the 34 kDa protein did not cross-react specifically with maize or spinach chloroplast thylakoid components (Ref. 25; see also Sherman, L.A. and Riethman, H.C., unpublished data). This antibody did, however, react with a protein of similar size in both the unrelated cyanobacterium *Synechocystis* sp. PCC6714 [16,25] and in *Prochlorothrix hollandica*, a Chl *b*-containing procaryote [26]. In each case, the reactive protein was of similar size and was associated with a Chl-protein complex, suggesting a similar evolutionary origin for these Chl-proteins.

Discussion

We have devised a procedure for the rapid separation of Chl-proteins from membranes of iron-stressed cells, and the purification of CPVI-4, the major Chl-protein present in these cells. Starting with membranes containing 9 mg Chl, we can isolate purified CPVI-4 material containing about 0.7 mg Chl within 36 h (the silver-stained polypeptide subunit composition of this sample is shown in Fig. 6, lanes 1 and 2). Much of the loss in yield of the first column is due to the presence of small amounts of CPVI-2 in the column fractions representing the right half of the main Chl peak in Fig. 1 (top). These fractions can be collected and re-chromatographed to recover more CPVI-4 from the same starting material.

The second DEAE-cellulose column was sometimes necessary to remove traces of CPVI-3 from the pooled peak fractions of the first column, and also to remove other minor contaminants. Depending on the resolution of the first column and the purpose of the experiment, this step may often be avoided. It leads to a further loss of material (about 0.25 mg Chl remains from the 0.7 mg Chl of CPVI-4 enriched material from the first column), partly because of irreversible binding of some sample to the second column and partly because of further denaturation of CPVI-4 during this step.

The asymmetric shape of the major CPVI-4 Chl peak (from both columns) has several plausible explanations. Native, maltoside-solubilized CPVI-4 might be present as two complexes comprised of differential amounts of the 36, 34 and 12 kDa proteins, or the 34 and 36 kDa proteins might

represent apoproteins of two separate Chl-protein particles having very similar ionic properties and sizes. We have not been able to resolve a green band containing only one or the other Chl-protein. Alternative explanations for the asymmetric Chl elution profiles of CPVI-4 from DEAE-cellulose include differential binding of lipids, Chl, or minor contaminating proteins to CPVI-4 complexes. A precursor-product relationship between the 36 kDa and 34 kDa proteins seems unlikely, but cannot be ruled out. The immunoblot data argue against structural similarities between the 34 and 36 kDa species, but several peculiar properties of the 36 kDa protein make these data difficult to interpret conclusively. The 36 kDa protein becomes a diffuse band upon denaturing LDS-polyacrylamide gel electrophoresis when solubilized at 70 °C, which would make immunodetection after blotting very difficult. Although the 36 kDa protein remains a relatively sharp band in gels when solubilized on ice, it transfers out of the gel poorly (as judged by amido black staining of the nitrocellulose and by silver-staining of post-blot gels). If the 34 kDa protein were an altered form of the 36 kDa protein, antibody recognizing the 34 kDa protein might not recognize the 36 kDa protein in either case. Further studies now possible with large quantities of purified CPVI-4 should help to resolve this question.

The 12 kDa protein is present in nearly stoichiometric quantities with the 34 kDa and the 36 kDa proteins in CPVI-4 (based on density of Coomassie-stained bands), but does not appear to bind Chl directly. Triton-X114 phase-partitioning exeriments indicated that the 12 kDa protein was hydrophobic (as were both the 34 and 36 kDa proteins; data not shown). The specific copurification of the 12 kDa protein with the 36 and 34 kDa proteins during chromatography strongly suggests that the three components were each part of the same submembrane particle.

The spectral properties of isolated CPVI-4 are typical of light-harvesting Chl-proteins [23,24]. Although immunologically unrelated to chloroplast LHC proteins, antibody reactive with the 34 kDa component of CPVI-4 was cross-reactive with a polypeptide of similar mol. wt. in the facultatively heterotrophic cyanobacterium *Synechocystis* sp. PCC6714 [16,25] and in the Chl *b*-containing procaryote, *Prochlorothrix hollandica* [26]. In both cases, the immunoreactive protein was associated with light-harvesting Chl-protein complexes. Thus, CPVI-4 appears to be one of a family of intrinsic, light-harvesting Chl-proteins found in procaryotes.

An unresolved problem in apparent conflict with the proposed light-harvesting function of CPVI-4 is the data obtained on the room temperature Chl fluorescence of iron-stressed cells [27,28]. In normally grown *A. nidulans* R2, the Chl fluorescence induction curve looks similar to that found in plant chloroplasts [28]. There is a moderate F_0 and a typical rise to F_{max} following blockage of electron flow to the plastoquinone pool with 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea. In iron stressed cells, however, there is a very large F_0 and no detectable F_v; the Chl fluorescence reaches its maximum almost instantaneously, and is not affected appreciably by the blockade of electron flow with 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea [4,27,28]. Upon recovery from iron stress, the fluorescence induction properties begin returning to normal within an hour or two and have returned to normal by about 10–12 h [27].

These fluorescence induction characteristics can be explained by a decrease in the number of trapping centers relative to the Chl pool feeding into them in iron-stressed cells. A comparison of the levels of PS II core proteins and CPVI-4 in iron-stressed cells and during recovery from iron stress indicates a large excess of CPVI-4 in iron-stressed cells (data not shown; see Ref. 8); much of the light absorbed by CPVI-4 would be immediately re-emitted as fluorescence, resulting in a very high F_0. Preliminary measurements of the PS I Chl absorption cross-section indicate that it increases only slightly in iron-stressed cells; this is in contrast to the 3–4-fold increase (from 200/1 to 650/1) of the Chl to reaction center ratios measured in the same samples (Melis, A. personal communication). A significant fraction of CPVI-4 might therefore be uncoupled or only inefficiently coupled to the photosystem core pigment beds. Öquist had noted a decreased quantum efficiency of electron transport through PS I as well as an increased Chl-to-P-700 ratio in iron-stressed *A. nidulans*; he suggested a decreased efficiency of

energy transfer within the PS I photosynthetic units [29]. We suggest the reason for the presence of excess Chl in these membranes is that CPVI-4 provides a membrane pool of Chl which is used to stabilize nascent CP apoproteins synthesized during recovery from iron stress.

Gabaculine specifically inhibits Chl biosynthesis in *A. nidulans* R2 [30], and when it was added along with iron to iron-stressed cultures, the typical decrease in the fluorescence yield (both F_0 and F_{max}) during recovery was not affected [27]. These experiments demonstrated that, as long as protein synthesis was allowed to occur, the spectral changes in room temperature Chl fluorescence upon recovery from iron stress (membrane development) occurred normally, even in the absence of new Chl. Guikema [27] concluded that recovery of this parameter was independent of Chl biosynthesis and likely dependent on repair or turnover of existing light-harvesting structures. Recent experiments in this laboratory (Bullerjahn, G.S., Troyan, T. and Sherman, L.A., unpublished results) indicate that the changes in 77 K Chl fluorescence emission associated with recovery from iron stress also occur in the presence of gabaculine. Since the 77 K Chl emission changes correlate closely with the accumulation of CP complexes typical of normally grown cells, these data strongly suggest that the Chl associated with CPVI-4 is transferred to other CP complexes during recovery from iron stress.

From immunoblotting experiments described previously [8,17], it is clear that the 34 kDa apoprotein of CPVI-4 is tightly regulated by iron. This regulation correlates closely with the accumulation pattern of the CPVI-4 holocomplex upon iron stress. The accumulation patterns of the 36 kDa and 12 kDa proteins during recovery from iron stress have not been established. The 36 kDa protein is particularly interesting, since it is enriched in the aggregated forms of CPVI-4 (Fig. 5) and displays unusual electrophoretic behavior. A 36 kDa protein has been detected in the high mol. wt. CP complexes CPIII and CPIV from normally-grown *A. nidulans* R2 [9], and the characteristic fuzzy staining pattern of the 36 kDa protein can be found upon silver staining of polypeptides from these green bands as well as in the polypeptide profile of partially purified PS I complexes after denaturing gel electrophoresis [8]. Other, less well characterized green lipoprotein complexes of *Anacystis* membranes prepared under a number of conditions also often contain a polypeptide with these properties (data not shown). A testable hypothesis is that this 36 kDa protein functions in the formation of CPIII and CPIV in normal cells, but is associated primarily with CPVI-4 in iron-stressed cells. It may actually bind very little Chl (or possibly none at all), yet could interact with each of the Chl-proteins of the thylakoid. The membrane development of *A. nidulans* R2 upon recovery from iron stress could provide a critical test of this hypothesis. The 36 kDa protein should be found in increasing abundance in CPIII and CPIV as recovery proceeds and the 34 kDa apoprotein of CPVI-4 diminishes.

The conditional accumulation of CPVI-4 in *A. nidulans* R2 and the genetic accessibility of this organism should facilitate future studies of this Chl-protein and its interactions with other thylakoid components. The availability of purified CPVI-4 in quantities suitable for biochemical and biophysical analyses of these interactions will allow a more detailed assessment of the evolutionary and functional questions raised by the existence of this unusual Chl-protein.

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