Photosynthesis Research **38**: 73–81, 1993.

Regular paper

Low molecular weight subunits associated with the cytochrome b_6f complexes from spinach and *Chlamydomonas reinhardtii*

Christian L. Schmidt[1] & Richard Malkin*
Department of Plant Biology, University of California, 111 Koshland Hall, Berkeley, CA 94720, USA;
*[1]Present address: Institut für Biochemie, Medizinische Universität zu Lübeck, Ratzeburger Allee 160, D-2400 Lübeck 1, Germany; *Author for correspondence/reprints*

Received 21 July 1993; accepted in revised form 8 August 1993

Keywords: cytochrome *f*, cytochrome b_6, thylakoid membranes, low molecular weight subunits, *petG*

Abstract

Cytochrome b_6f complexes, prepared from spinach and *Chlamydomonas* thylakoids, have been examined for their content of low molecular weight subunits. The spinach complex contains two prominent low molecular weight subunits of 3.7 and 4.1 kD while a single prominent component of 4.5 kD was present in the *Chlamydomonas* complex. An estimation of the relative stoichiometry of these subunits suggests several are present at levels approximating one copy per cytochrome complex. The low molecular weight subunits were purified by reversed phase HPLC and N-terminal sequences obtained. Both the spinach and *Chlamydomonas* cytochrome complexes contain a subunit that is identified as the previously characterized *petG* gene product (4.8 kD in spinach and 4.1 kD in *Chlamydomonas*). A second subunit (3.8 kD in spinach and 3.7 kD in *Chlamydomonas*) appears to be homologous in the two complexes and is likely to be a nuclear gene product. The possible presence of other low molecular weight subunits in these complexes is also considered.

Introduction

The cytochrome b_6f complex from the photosynthetic membranes of higher plants has been intensively studied since the first description of its isolation by Hurt and Hauska (1981). Like the closely related cytochrome bc_1 complexes of mitochondrial and photosynthetic bacterial membranes, it catalyses the electron transfer from a quinol (plastoquinol in higher plants) to a soluble single electron acceptor (plastocyanin in higher plants or soluble c-type cytochromes in cyanobacteria). Thus, in the case of higher plants, this complex may be described as a plastoquinol-plastocyanin oxidoreductase. The energy from this redox reaction is used to establish a proton gradient across the membrane that is subsequently used for the synthesis of ATP (Hurt et al. 1983). Many structural and functional similarities are found between the cytochrome b_6f and the bc_1 complexes, and the basic reaction mechanism of the Q-cycle is believed to be the same in both cases (Hauska 1986, Rich 1986, Malkin 1988).

One main difference between the photosynthetic cytochrome b_6f and the mitochondrial cytochrome bc_1 complexes is the relative complexity of the enzymes in terms of protein subunit composition. While the cytochrome bc_1 complex from beef heart contains eleven subunits (Ljungdahl et al. 1987, Malkin 1988), only four major subunits have been found for the cytochrome b_6f complex from the thylakoid membranes of spinach and other higher plants (Malkin 1988, Widger and Cramer 1991). Three of these carry prosthetic groups that participate in the redox reactions (the cytochromes *f* and b_6 and the Rieske iron sulfur protein) while the fourth (subunit IV) is believed to be involved in the binding of plastoquinone (Li et al. 1991). Two large core proteins and several smaller proteins with molecular

weights between 15 and 6.4 kD are also found in the cytochrome bc_1 complex from beef heart (see Malkin 1988 for a comparative review). None of these additional subunits contain prosthetic groups and two of the small proteins are found in tight association with cytochrome c_1 (Schagger et al. 1987, Wakabayashi et al. 1982). It is interesting to note that recent studies have identified several low molecular weight subunits, mostly of unknown function, in the reaction center complexes of the photosynthetic membranes of higher plants and cyanobacteria (Ikeuchi et al. 1989, 1990). Very little is known about the presence of low molecular weight subunits associated with the cytochrome b_6f complex. The presence of such subunits was first noted in early work by Hurt and Hauska (1982), but little characterization of these subunits was done in this work. In cross linking studies, Lam (1986) observed two low molecular weight proteins of 13 and 9.5 kD in purified cytochrome b_6f preparations of which the 9.5 kD protein proved to be very sensitive to cross linking with glutaraldehyde. Anderson (1986) mentions a 5 kD protein of unknown function as fifth subunit of the cytochrome b_6f complex. Haley and Bogorad (1989) observed four low molecular weight proteins (6.0, 4.0, 3.6 and 3.2 kD) in purified cytochrome b_6f preparations from spinach thylakoids. Using an antibody directed against a synthetic decapeptide, they identified the 4.0 kD protein as the product of the plastid *petG* gene (*petE* in the nomenclature of Haley and Bogorad). They were able to demonstrate the presence of this protein in the thylakoid membranes of other higher plants but not in the green algae, *Chlamydomonas reinhardtii*, and the cyanobacterium, *Synechocystis 6803*. The *petG* gene has been detected in many higher plants (corn, tobacco, rice and peas) (Haley and Bogorad 1989) as well as in *Marchantia polymorpha* (Haley and Bogorad 1989), the cyanelles of *Cyanophora paradoxa* (Stirewalt and Bryant 1989) and, while this work was in progress, in *Chlamydomonas reinhardtii* (Fong and Surzycki 1992).

Using a new developed and very effective method for the purification of the cytochrome b_6f complex, we investigated the low molecular weight proteins copurifying with the cytochrome b_6f complexes from spinach and *Chlamydomonas reinhardtii*. We are able to confirm the presence of three low molecular weight proteins (including the *petG* protein) in the purified spinach cytochrome b_6f complex. For two of these, a homologous, or very similar protein, was detected in the purified cytochrome b_6f complex of *Chlamydomonas reinhardtii*.

Materials and methods

Biological material

Spinach was purchased at the local market or greenhouse-grown. *Chlamydomonas reinhardtii*, strain cc-124 was grown on a Tris- acetate-phosphate medium (Harris 1989).

Antibodies

An antibody against the C-terminal portion of the *petG* protein was a gift from Dr J. Haley and Dr L. Bogorad.

Other materials

N-propyl agarose, Sephacryl S 400 HR and n-decyl plastoquinone were purchased from Sigma Chemical Company. Dodecylmaltoside was from Boehringer Mannheim. Plastocyanin was purified from spinach by R. K. Chain from our laboratory.

Electrophoretic methods

Tris-Tricine-SDS-PAGE was done by the method described by Schagger and von Jagow (1987) with the modifications described by Zilber and Malkin (1992). 1 mm gels were used. The samples were electrophoresed overnight at room temperature. The voltage was limited to 130 V and the current to 65 mA.

Quantitation of the stained gels was performed using a UltroScan XL laser densitometer and the GelScan XL software package (Pharmacia LKB, Piscataway, N.J.).

Immunoblotting was carried out as described by Zilber and Malkin (1992) using the horseradish peroxidase system.

Purification of the cytochrome b_6f *complex*

Thylakoid membranes from spinach were isolated and treated with NaBr as described by Willms et al.

(1987). The cytochrome b_6f complex from spinach thylakoid membranes was purified as follows: The NaBr treated thylakoid membranes were resuspended at a concentration of 2 mg chlorophyll ml^{-1} in the solubilization buffer containing: 25 mM Tris-HCl, pH 7.5; 1 mM $MgCl_2$; 15 mM dodecylmaltoside (DM) and 10% (w/v) ammonium sulphate. The solubilization mixture was incubated for 30 min at room temperature in the dark and subsequently spun at 4 °C for 60 min at 60 000 rpm in a Beckman 60 Ti rotor. All following steps were carried out at 4 °C. A saturated ammonium sulphate solution (pH 7.5) was added to the supernatant (218 ml, containing 539 nmol cytochrome *f*) to achieve 35% saturation. Precipitated material was removed by a short centrifugation (15 min at 12 000 rpm in a Beckman JA-14 rotor). The supernatant (268 ml, 538 nmol cytochrome *f*) was loaded at a flow rate of 1 ml min^{-1} onto a propyl agarose column (diameter =1.5 cm, length =11.3 cm) equilibrated with 25 mM Tris-HCl, pH 7.5; 1 mM $MgCl_2$; 10 mM DM and 35% saturated ammonium sulphate. Subsequently the column was washed with 160 ml of the same buffer. The cytochrome b_6f complex was eluted with a total volume of 50 ml of 25 mM Tris-HCl, pH 7.5; 1 mM $MgCl_2$; 0.3 mM DM and 20% saturated ammonium sulphate.

The fractions with a cytochrome *f* concentration higher than 3 μM were pooled (35 ml, 428 nmol cytochrome *f*), concentrated on a YM 100 membrane (Amicon) to a final volume of 15 ml and loaded at a flow rate of 0.25 ml min^{-1} onto a Sephacryl S 400 HR column (length = 51 cm, diameter = 2.5 cm) equilibrated and eluted with: 25 mM Tris-HCl, pH 7.5; 1 mM $MgCl_2$ and 0.2 mM DM. A small amount (approx. 15%) of the cytochrome b_6f complex eluted together with the chlorophyll containing proteins prior to and well separated from the main fraction.

The fractions from the main peak with cytochrome *f* concentrations higher than 1 μM (15 ml, 233 nmol cytochrome *f*) were pooled and loaded at a flow rate of 0.5 ml min^{-1} onto a hydroxyapatite column (length = 4 cm, diameter = 2.5 cm) equilibrated with the same buffer as the Sephacryl S 400 HR column. The column was subsequently washed with 60 ml equilibration buffer, 100 ml 0.4 M potassium phosphate dissolved in equilibration buffer and 10% saturated ammonium sulphate dissolved in equilibration buffer. The purified cytochrome b_6f complex was eluted with 20% saturated ammonium sulphate dissolved in equilibration buffer (28.3 ml, 208 nmol cytochrome *f*). Aliquots were immediately frozen in liquid nitrogen and stored at –80 °C.

Thylakoid membranes from *Chlamydomonas reinhardtii* were isolated as previously described (Wynn et al. 1988). The cytochrome b_6f complex from *Chlamydomonas reinhardtii* was purified using the chromatographic method described above, or for comparison, by the method of Hurt and Hauska (1981) using octylglucoside, modified as described by Wynn et al (1988).

HPLC purification of the small proteins associated with the cytochrome b_6f *complex*

Sample preparation

2–5 nmol of the purified b_6f complex were concentrated on an Ultrafree-MC (Millipore) membrane with a cut off of 100 KD until the membrane appeared to be dry. The protein was resuspended using 400 μl of 25 mM Tris-HCl pH 7.5 and spun to dryness again. It was dissolved in 100 μl of glacial acetic acid and diluted by addition of 400 μl 70% aqueous acetonitrile. The solution was spun for 15 min in an Eppendorf centrifuge (at 4 °C) and stored on ice until it was injected into the column.

HPLC separations

The first step was a gel filtration on a TSK G-2000 SW, 7.6 mm × 600 mm, column (Pharmacia LKB, Piscataway, N.J.) using 70% acetonitrile; 0.1% trifluoro acetic acid (TFA) as solvent. The flow rate was 0.3 ml / min. 500 μl of sample were injected. The eluting protein was monitored at 210 nm and fractions were collected manually.

The fractions containing the small proteins (as determined by Tris-Tricine SDS-PAGE) were dried down on the speed vac, dissolved in a minimal volume of TFA (5 μl) and diluted by addition of 100 μl of 50% acetonitrile. The solution was spun in the Eppendorf centrifuge for 15 min and loaded onto a reversed phase HPLC column (SynChropak, reversed phase, C1, 300 Å, 50 mm × 4.6 mm, SynChrom Inc.) The column was eluted with a gradient of 0 to 100% acetonitrile with 0.1% TFA as modifier. The fractions were collected manually and assayed for the small proteins by Tris-Tricine SDS-PAGE.

Amino acid sequencing

N-terminal sequencing of the purified small peptides was done by automated Edman degradation at the micro chemical facility of the University of California, Berkeley.

Other methods

The concentrations of the cytochromes b_6 and f were measured spectrophotometrically as previously described (Willms et al. 1987). The activities of the cytochrome b_6f complexes were measured by following the reduction of spinach plastocyanin at 600–500 nm using a SLM-Aminco spectrophotometer as described by Chain (1985) with slight modifications: 0.2 mM DM was added to the assay medium and 5 μM n-decyl plastoquinol was used as substrate. N-decyl plastoquinone was reduced with $NaBH_4$ as described by Chain (1985).

Results

Purification and subunit composition of the cytochrome b_6f *complex*

Figure 1a shows a Tris-Tricine-SDS gel of the spinach cytochrome b_6f complex at different stages of its purification. Lane 4 shows the purified complex after the last purification step. Two of the subunits, cytochrome b_6 and the Rieske iron sulfur protein, comigrate with a molecular weight of approximately 20 kD in this gel system and appear unresolved. However, electrophoresis on a Laemmli SDS-PAGE gel confirmed the presence of the Rieske iron sulfur protein in this preparation (data not shown). The cytochrome b_6:f ratio of this preparation was 1.93; the activity was 92 μmol plastocyanin reduced per nmol cytochrome f per h. Figure 2, lane 3, shows the cytochrome b_6f complex from *Chlamydomonas reinhardtii* purified by the same procedure. In this case, the final preparation contained several contaminating subunits, but the b_6:f ratio of this preparation was 1.98 and the activity was 45 μmol plastocyanin reduced per nmol cytochrome f per h.

Figure 1b shows densitometer traces of the low molecular weight region of the gel shown in Fig. 1a. The gel, as well as the densitometer traces, show that two pronounced low molecular weight bands with apparent molecular weights of 3.7 and 4.1 kD as well as two weaker bands with apparent molecular weights of 4.8 and 5.3 kD are copurifying with the four known larger subunits. These four low molecular weight proteins were also detected in preparations purified by the method of Hurt and Hauska (1981,

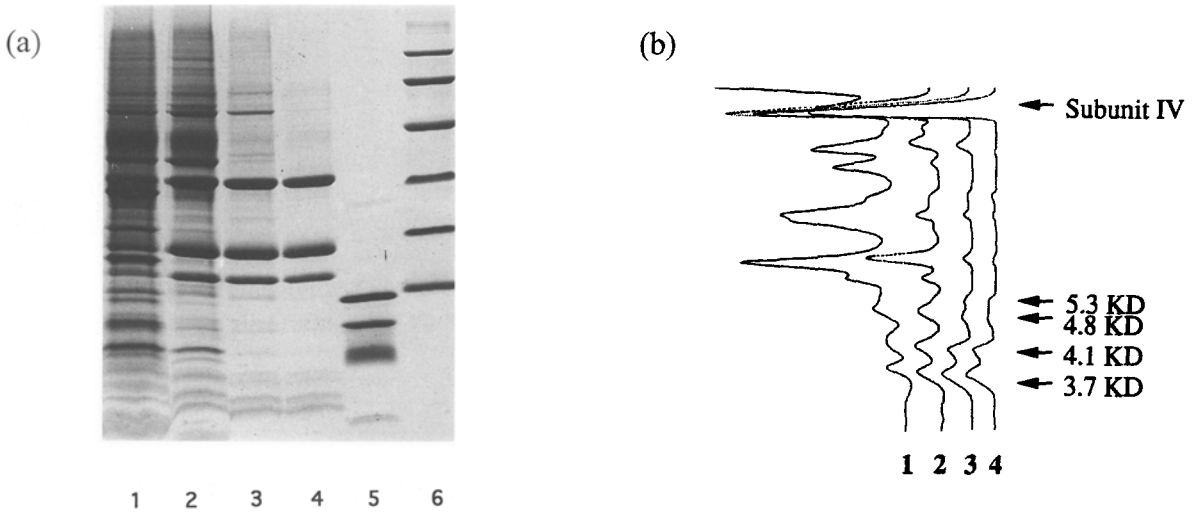

Fig. 1. Tris-Tricine-SDS gel (a) of the spinach cytochrome b_6f complex at different stages of purification and (b) densitometer traces of the lower part of the gel. (a) Lane 1: Detergent extract, 0.2 nmol cytochrome f; lanes 2, 3, and 4: cytochrome b_6f complex purified over the propyl agarose, Sephacryl S 400 HR, and hydroxyapatite columns; 0.5 nmol cytochrome f were loaded on each lane. Lane 5: low molecular weight markers: cytochrome c, 12.4 kD; plastocyanin, 10.5 kD; aprotinin, 6.5 kD and glucagon, 3.5 kD. Lane 6: high molecular weight markers: 97.4 kD, 66.2 kD, 42.7 kD, 31.0 kD, 21.5 kD, and 14.4 kD. (b) Densitometer traces of the corresponding lanes of the gel shown in (a). Only the lower part of the traces is shown in order to demonstrate the presence of the low molecular weight bands at all steps of the purification.

1982) (data not shown).

The results were less clear in the case of the cytochrome b_6f complex isolated from *Chlamydomonas*. However, comparison of different preparations revealed a band with an apparent molecular weight of 4.5 kD copurifying with the four large subunits in all cases (Fig. 2). Assuming that the staining intensity of a protein band on the gel is solely a function of the amount and the apparent molecular weight of protein present in the band, an assumption we realize is not perfectly correct, we calculated stoichiometries for the subunits of the cytochrome b_6f complexes from spinach and *Chlamydomonas* including the low molecular weight proteins. Typical results expressed as the number of protein subunits per one copy of subunit IV are given in Table 1. Subunit IV was used as reference because it showed an average staining intensity in the spinach complex and it was the band that showed the best resolution in all stages of purification. The values for the large subunits agree fairly well with the known stoichiometry of 1:1:1:1 with exception of the *Chlamydomonas* cytochrome b_6 which seems to have higher staining intensity than cytochrome *f* or subunit IV. For the low molecular weight proteins, these results suggest that at least the 3.7 kD and the 4.1 kD proteins from spinach are present in a 1:1 ratio with subunit IV. The intensity of the 4.5 kD band associated with the cytochrome b_6f complex from *Chlamydomonas* may indicate the presence of two low molecular weight proteins (either identical or two different ones) per subunit IV. It should be noted that the *Chlamydomonas* cytochrome b_6f complex, isolated by the modified method of Wynn et al. (1988), showed a substantial depletion of the Rieske iron sulfur protein, whereas the amount of the low molecular weight proteins showed no significant difference when compared with other preparations that were not depleted of the Rieske iron-sulfur protein (Fig. 2, Table 1).

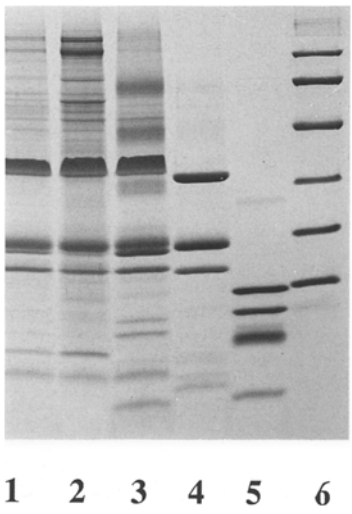

Fig. 2. Tris-Tricine-SDS gel of the cytochrome b_6f complex isolated from *Chlamydomonas reinhardtii*. Lanes 1 and 2: cytochrome b_6f complex purified by ammonium sulphate precipitation followed by sucrose density gradient centrifugation. Lane 3: cytochrome b_6f complex isolated by the chromatographic method as described under 'Materials and methods'. Lane 4: spinach cytochrome b_6f complex. 0.5 nmol cytochrome *f* were loaded on each lane. Lanes 5 and 6: molecular weight markers as in Fig. 1. The band migrating with an apparent molecular weight of less than 3.5 kD (lane 3) contains the chlorophyll still present in this preparation.

Figure 3 shows a Western blot using the *petG* antibody and the corresponding gel with the cytochrome b_6f complexes from spinach and *Chlamydomonas*. No cross reaction could be detected with any of the proteins of the *Chlamydomonas* cytochrome complex, whereas the 4.8 kD and the 5.3 kD band of the spinach complex reacted equally with

Table 1. Stoichiometries of subunits in the cytochrome b_6f complexes from spinach and *Chlamydomonas reinhardtii*. The stoichiometries of the subunits were calculated from the staining intensities of the bands after SDS-PAGE analysis. The values for subunit IV were normalized to 1.0 for each analyzed lane

Subunit isolated from	Cytochrome *f*	Cytochrome b_6 and RFeS	Subunit IV	5.3 and 4.8 KD band	4.5 KD band	4.1 KD band	3.7 KD band
Spinach	1.3	1.8	1.0	0.2		0.5	0.7
Chlamydomonas[a]	1.3	2.9	1.0		1.6		
Chlamydomonas[b]	1.3	2.2	1.0		1.5		

[a] Prepared by the chromatographic method described under 'Materials and methods'.
[b] Prepared by ammonium sulphate precipitation and sucrose density gradient centrifugation as described by Hurt and Hauska (1982).

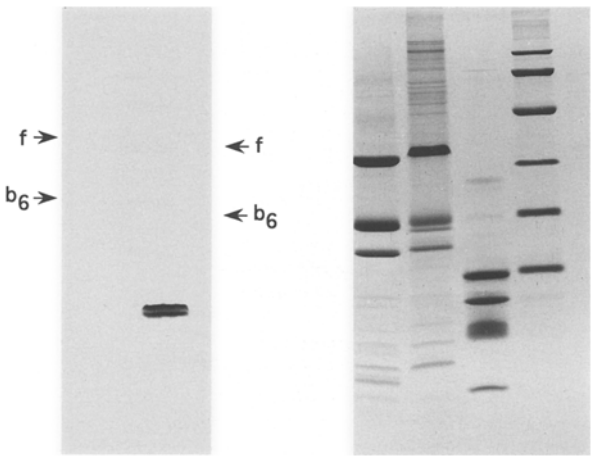

Fig. 3. Western blot and the corresponding Tris-Tricine-SDS of the cytochrome b_6f complexes isolated from spinach and *Chlamydomonas reinhardtii*. The antibody was directed against a synthetic peptide representing the amino acids positions 28–37 of the *petE* protein from corn (Haley and Bogorad 1989). Lanes 1 and 4: 0.5 nmol cytochrome b_6f complex isolated from *Chlamydomonas reinhardtii*. Lanes 2 and 3: 0.5 nmol cytochrome b_6f complex isolated from spinach. Lanes 5 and 6: molecular weight markers as in Fig. 1, lanes 5 and 6. The arrows indicate the positions of the cytochromes b_6 and f on the blot.

the antibody. It is probable the low molecular weight subunit is a proteolytic product although sequence analysis would be required to confirm this result.

Purification and sequencing of the low molecular weight proteins

Figure 4a and b shows the purification of the small proteins associated with *Chlamydomonas* cytochrome b_6f complex. Two compounds with apparent molecular weights of 3.8 kD (Csp 3.8) and 4.1 kD (Csp 4.1) could be separated on the reversed phase column. Figure 4c shows the low molecular weight proteins of the spinach cytochrome b_6f complex as they eluted from the reversed phase column. The 3.7 kD (Sp 3.7) and the 4.8 kD (Sp 4.8) proteins were usually obtained almost pure. The 4.1 kD protein (Sp 4.1) was frequently contaminated with one or both of the other proteins and was further purified by repeating the chromatography on the reversed phase column (not shown). No trace of a 5.3 kD protein could be detected in the fractions from the reversed phase column.

N-terminal amino acid sequences could be obtained for Sp 3.7 and Sp 4.8 from spinach as well as for Csp 4.1 and Csp 3.8 from the *Chlamydomonas*

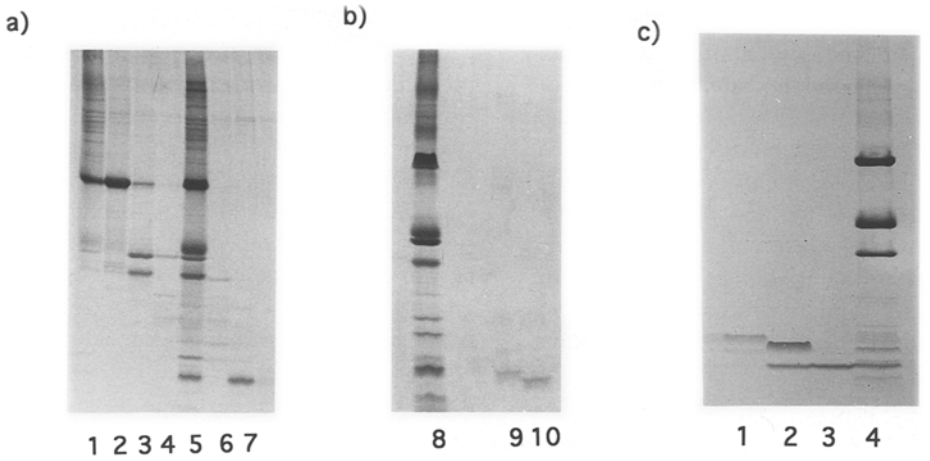

Fig. 4. Purification of the low molecular weight proteins associated with the cytochrome b_6f complex from *Chlamydomonas reinhardtii* (Fig. 4a,b) and spinach (Fig. 4c). Separation of the large and the small proteins of the *Chlamydomonas reinhardtii* cytochrome b_6f complex by HPLC gel permeation chromatography (a) and separation of the two small proteins by chromatography on the reversed phase C1 column (b). Lanes 5 and 8: 0.2 nmol of the cytochrome b_6f complex. Lanes 1 to 4 and 6,7: fractions eluted from the gel permeation column. Lane 7 shows the small proteins. Lane 9: purified 4.1 kD protein (Csp 4.1) with a minor contamination. Lane 10: purified 3.8 kD protein (Csp 3.8). Lanes 9 and 10 approximately 0.4 μg protein were loaded on each lane. (c) Separation of the small proteins associated with the spinach b_6f complex on the reversed phase HPLC column. Lane 1: 4.8 kD protein (Sp 4.8) slightly contaminated by the 4.1 kD protein. Lane 2: A fraction containing the 4.1 kD protein (Sp 4.1) and some of the 3.7 kD protein. Lane 3: The pure 3.7 kD protein (Sp 3.7). Lane 4: 0.5 nmol of the spinach cytochrome b_6f complex.

complex. From comparison with known protein or DNA sequences, as shown in Fig. 5, it is obvious that Sp 4.8 and Csp 4.1 are the products of the spinach and *Chlamydomonas petG* genes, respectively.

No proteins with striking similarities to the Csp 3.8 and the Sp 3.7 proteins could be found during an intensive data bank search. This is of special importance because the complete chloroplast DNA sequences of tobacco, rice and liverwort were included in this search. Thus, Csp 3.8 and Sp 3.7 are encoded by the nuclear DNA. Both sequences show some similarity to each other (45% identity and some conservative replacements) if they are aligned as shown in Fig. 5. In addition, they may have a similar secondary structure since hydropathy plots indicate a more hydrophilic N-terminus and the beginning of a hydrophobic helix (Fig. 6). No conclusive sequence data could be obtained for the Sp 4.1 protein, most likely due to a blocked N-terminus.

a)

```
                      1    5        10        15        20
Corn:                 MIEVF LFGIV LGLIP ITLAG
Sp 4.8 (Spinach):     MIEVF LFGIV LGLIG ITLAG
Chlamydomonas:        MVEPL LCGIV LGLVP VTIAG
Csp 4.1:              MVEPL LLGIV LGLVP
Liverwort:            MVEAL LSGIV LGLIP ITLLG
Cyanophora:           MVEPL LSGIV LGLIP VTLIG
```

b)

```
                          1    5        10        15        20
Csp 3.8 (Chlamydomonas):  GEAEF IAGTA LTMVG MTLVG LA
Sp 3.7 (Spinach):         N AAAEI F?IAA V-MNG LTLVG
```

Fig. 5. Alignment and sequence comparison of the *petG* protein sequences (a) and the partial sequences of the Csp 3.8 and the Sp 3.7 proteins (b). The *petG* sequences are from: corn and liverwort: protein sequences deduced from the genes (Haley and Bogorad 1989); *Chlamydomonas*: protein sequence deduced from the gene (Fong and Surzycki 1992); *Cyanophora*: protein sequence deduced from the gene (Stirewalt and Bryant 1989); and Csp 4.1 and Sp 4.8: sequences obtained by N-terminal sequencing (this report). Residues that were identical in all analyzed sequences are shown bold. (?): Residue not identified; (-): Gap introduced for better alignment.

Discussion

In this report we present strong evidence for the presence of low molecular weight subunits in the cytochrome b_6f complexes from spinach and the green algae *Chlamydomonas reinhardtii*. Three small proteins were found to copurify with the spinach complex and at least two with the cytochrome b_6f complex of *Chlamydomonas*. The fourth band observed on the Tris-Tricine-SDS gel of the spinach complex is likely to be an artifact due to incomplete denaturation of the complex. No trace of a 5.3 kD protein was detected during the HPLC purifications. Instead, the recovery of the Sp 4.8

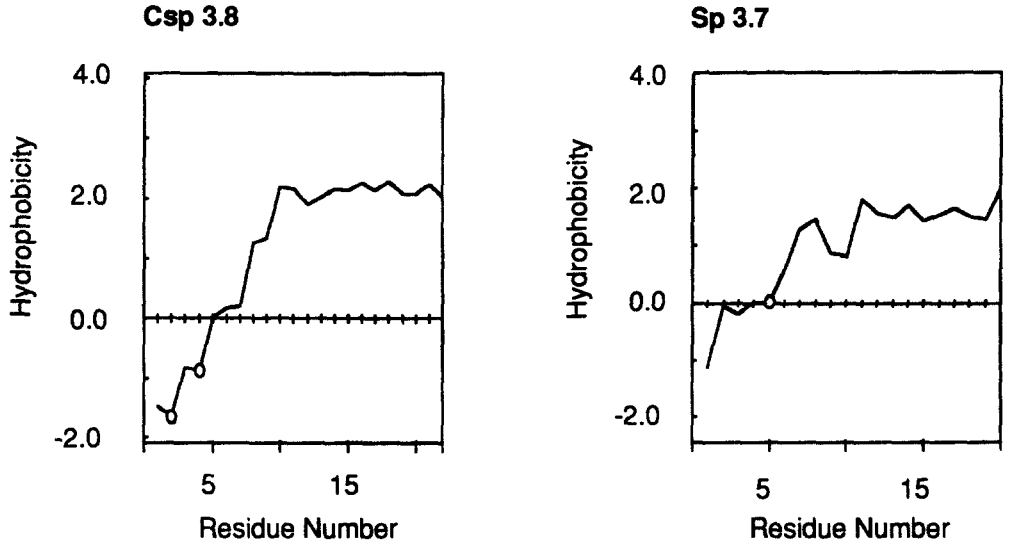

Fig. 6. Hydropathy plots of the partial sequences of the Csp 3.8 and the Sp 3.7 proteins. The averaging window was 11 residues, and positive values represent more hydrophobic amino acids. The circles indicate the glutamate residues. Both plots show evidence for the presence of hydrophobic and possibly membrane spanning helices.

protein was higher than expected (not shown). Further evidence for the identity of the 4.8 kD and the 5.3 kD bands comes from the reaction of both bands with the *petG* antibody (Fig. 3).

Contrary to the immunological evidence presented by Haley and Bogorad (1989), we were able to document the presence of the *Chlamydomonas petG* protein and show its association with the cytochrome b_6f complex in this organism. Since the amino acids in positions 34–37 show a great difference between the corn (Haley and Bogorad 1989, also see Fig. 5) and the *Chlamydomonas* protein, it is not surprising that an antibody directed against a synthetic polypeptide representing the positions 28–37 of the corn protein shows no reactivity with the *Chlamydomonas* protein. The discrepancy between position 7 of the sequence obtained by Edmann degradation and the amino acid sequence deduced from the DNA sequence (Fong and Surzycki 1992) is likely to be an artifact from the protein sequencing. The cysteine at position 7 was confirmed by DNA sequencing in our lab (D. Berthold, personal communication). The complete match of the other positions provides strong evidence for the identity of the protein.

Our results support the hypothesis of Haley and Bogorad (1989) that the *petG* protein is indeed a fifth subunit of the cytochrome b_6f complex. The occurrence of the protein in *Chlamydomonas* and of the homologous gene in the cyanelles of *Cyanophora paradoxa* (Stirewalt and Bryant 1989) suggests that the *petG* protein is not exclusively found in higher plants and green algae, but may be present in cyanobacteria as well.

Our results on the Sp 3.7 protein from spinach and the Csp 3.8 protein from *Chlamydomonas* indicate the presence of a possible sixth subunit in the cytochrome b_6f complex. Like the *petG* protein, this subunit is tightly associated with the complex. The results presented in Fig. 2 and Table 1 indicate that, at least in the case of the *Chlamydomonas* complex, the interactions between the low molecular weight subunits and the cytochromes and subunit IV are stronger than the interactions between the Rieske iron sulfur protein and the other large proteins. The hydropathy plots of the *petG* protein shown by Haley and Bogorad (1989) indicate the presence of a membrane spanning helix. The hydropathy plots of the fragments of the Csp 3.8 and Sp 3.7 proteins show evidence for the existence of a similar secondary structure (Fig. 6). Thus the low molecular weight subunits are probably interacting with the large ones by strong hydrophobic interactions. The sequence similarities, the similar secondary structure and the surprising fact that both are nuclear encoded suggest that the Csp 3.8 and the Sp 3.7 proteins may be homologues. Further investigation will be necessary to clarify this point.

At the present time we have no detailed information about the third low molecular weight protein (Sp 4.1) found in the spinach complex. From the current results, it seems to be clear that it is different from the Sp 3.7 and Sp 4.8 proteins and may be another subunit of the spinach cytochrome b_6f complex.

The stoichiometry for the subunits of the cytochrome b_6f complex, including the small subunits, remains unclear. The values calculated in Table 1 suggest that the *petG* protein in the spinach complex is present in a ratio of less than 1 per b_6f monomer. However, the method used to obtain these ratios is sensitive to errors. It is well known that the amino acid composition of a protein has an influence on the staining intensity of the protein. This effect should be much more pronounced in the case of a small protein. Also, the rapid diffusion of small proteins in combination with the possible solubility of membrane proteins in organic solvents may be reasons for an underestimation of the protein amount. A possible stoichiometry would be one *petG* subunit per cytochrome b_6f monomer or dimer.

The other low molecular weight proteins (Sp 3.7, Csp 3.8 and Sp 4.1) seem to be more abundant. Not only the data shown in Table 1, but also the elution profiles from the HPLC separations of the small proteins, indicate that two Csp 3.8 or Sp 3.7 are present per one Csp 4.1 or Sp 4.8 (data not shown). An overall stoichiometry of 0.5–1 *petG* protein and 1–2 proteins of Csp 3.8 (in *Chlamydomonas*), or each of Sp 3.7 and Sp 4.1 (in spinach) seems most likely.

There are presently no indications as to the function of these newly identified subunits. Since the *petG* protein is well conserved in higher plants and organisms as distant as *Cyanophora paradoxa*, it can be assumed that it is an essential component in the functioning of the cytochrome b_6f complex.

Acknowledgements

We wish to thank R. K. Chain for technical assistance and helpful discussions as well as for supplying the spinach plastocyanin. Helpful comments from Dr A. L. Zilber are gratefully acknowledged. Thanks are due to M. Moore and S. Kadambi for their help with the HPLC separations and for the protein sequencing. This work was supported by a grant from the National Institutes of Health (GM-20571) to R. M.

References

Anderson JM (1986) Photoregulation of the composition, function, and structure of thylakoid membranes. Ann Rev Plant Physiol 37: 93–136

Chain RK (1985) Involvement of plastoquinone and lipids in electron transport reactions mediated by the cytochrome b_6f complex isolated from spinach. FEBS Lett 180: 321–325

Fong SE and Surzycki SJ (1992) Organization and structure of plastome *psbF*, *psbL*, *petG* and ORF712 genes in *Chlamydomonas reinhardtii*. Curr Gen 21: 527–530

Haley J and Bogorad L (1989) A 4-kDa maize chloroplast polypeptide associated with the cytochrome b_6f complex: Subunit 5, encoded by the chloroplast *petE* gene. Proc Natl Acad Sci USA 86: 1534–1538

Harris EH (1989) The *Chlamydomonas* Sourcebook. Academic Press, San Diego

Hauska G (1986) Composition and structure of cytochrome *bc* and b_6f complexes. In: Staehelin A and Arntzen CJ (eds) Photosynthesis III. Photosynthetic Membranes and Light Harvesting Systems, pp 496–507. Springer-Verlag, Berlin

Hurt E and Hauska G (1981) A cytochrome f/b_6 complex of five polypeptides with plastoquinol-plastocyanin oxidoreductase activity from spinach chloroplasts. Eur J Biochem 117: 591–599

Hurt E and Hauska G (1982) Identification of the polypeptides in the cytochrome b_6f complex from spinach chloroplasts with redox-carrying subunits. J Bioenerg Biomembr 14: 119–138

Hurt EC, Gabellini N, Shahak Y, Lockau W and Hauska G (1983) Extra proton translocation and membrane potential generation: Universal properties of cytochrome bc_1/b_6f complexes reconstituted into liposomes. Arch Biochem Biophys 225: 879–885

Ikeuchi M and Inoue Y (1989) Identification of *psbI* and *psbL* in cyanobacterial Photosystem II reaction center preparation. FEBS Lett 251: 155–160

Ikeuchi M, Hirano A, Hiyama T and Inoue Y (1990) Polypeptide composition of higher plant photosystem I complex: Identification of *psaI*, *psaJ*, and *psaK* gene products. FEBS Lett 263: 274–278

Lam E (1986) Nearest-neighbor relationships of the constituent polypeptides in plastoquinol-plastocyanin oxidoreductase. Biochim Biophys Acta 848: 324–332

Li L-B, Zou Y-P, Yu L and Yu C-A (1991) The catalytic role of subunit IV of the cytochrome b_6f complex from spinach chloroplast. Biochim Biophys Acta 1057: 215–222

Ljungdahl P O, Pennoyer J D, Robertson D E and Trumpower B L (1987) Purification of highly active cytochrome bc_1 complexes from phylogenetically diverse species by a single chromatographic procedure. Biochim Biophys Acta 891: 227–241.

Malkin R. (1988) Structure-function studies of photosynthetic cytochrome bc_1 and b_6f complexes. In: ISI Atlas of Science, pp 57–64. Institute for Scientific Information, Philadelphia

Rich PR (1986) A perspective on Q-cycles. J Bioenerg Biomembr 18: 145–156

Schägger H and von Jagow G (1987) Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal Biochem 166: 368–379

Stirewalt VL and Bryant DA. (1989) Molecular cloning and nucleotide sequence of the *petG* gene of the cyanelle genome of *Cyanophora paradoxa*. Nucleic Acids Res 17: 10095–10095

Wakabayashi S, Takeda H. Matsubara H, Kim CH and King TE (1982) Identity of the heme-not-containing protein in bovine heart cytochrome c_1 preparation with the protein mediating c_1-*c* complex formation – a protein with high glutamic acid content. J Biochem 91: 2077–2085

Widger WR and Cramer WA (1991) The Cytochrome b_6f Complex: In: Bogorad L and Vasil IK (eds) Cell Culture and Somatic Cell Genetics of Plants, Vol 7B: The Molecular Biology of Plastids, pp 149–176. Academic Press, San Diego

Willms I, Malkin R and Chain RK (1987) Oxidation-reduction reactions of cytochrome b_6 in a liposome-incorporated cytochrome b_6f complex. Arch Biochem Biophys 258: 248–258

Wynn RM, Bertsch J, Bruce BB. and Malkin R (1988) Green algal cytochrome b_6f complexes: Isolation and characterization from *Dunaliella salina*, *Chlamydomonas reinhardtii* and *Scenedesmus obliquus*. Biochim Biophys Acta 935: 115–122

Zilber AL and Malkin R (1992) Organization and topology of photosystem I subunits. Plant Physiol 99: 901–911