Electron Transfer and Stability of the Cytochrome b6f Complex in A Small Domain Deletion Mutant of Cytochrome f

by

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Running title: The small domain deletion mutant of cytochrome f
SUMMARY

The lumen segment of cytochrome f consists of a small and a large domain. The role of the small domain in the biogenesis and stability of the cytochrome b6f complex and electron transfer through the cytochrome b6f complex was studied with a small domain deletion mutant in *Chlamydomonas reinhardtii*. The mutant is able to grow photoautotrophically but with a slower rate than the wild type strain. The heme group is covalently attached to the polypeptide and the visible absorption spectrum of the mutant protein is identical to that of the native protein. The kinetics of electron transfer in the mutant were measured by flash kinetic spectroscopy. Our results show that the rate for the oxidation of cytochrome f was unchanged (t1/2: ca.100 µs), but the half time for the reduction of cytochrome f is increased (32 ms, wild type: 2.1 ms); Cytochrome b6 reduction was slower than that of the wild type by a factor of 2 approximately (t1/2 : 8.6 ms, wild type: 4.7 ms); The slow phase of the electrochromic band shift also displayed a slower kinetics (t1/2: 5.5 ms, wild type: 2.7 ms). The stability of the cytochrome b6f complex in the mutant was examined by following the kinetics of the degradation of the individual subunits after inhibiting protein synthesis in the chloroplast. The results indicate that the cytochrome b6f complex in the small domain deletion mutant is less stable than in the wild type. We conclude that the small domain is not essential for the biogenesis of cytochrome f and the cytochrome b6f complex. However, it does have a role in electron transfer through the cytochrome b6f complex and contributes to the stability of the complex.
INTRODUCTION

Functioning as a plastoquinone-plastocyanin oxidoreductase, the cytochrome b6f complex transfers electrons from PSII to PSI in the photosynthetic electron transfer chain of all oxygen-evolving organisms. Cytochrome f is one of the four redox centers and the largest subunit (31 kDa) in the cytochrome b6f complex. It transfers electrons from the Rieske FeS protein to plastocyanin. The biogenesis of cytochrome f and the assembly of the cytochrome b6f complex involve a complicated process (1, 2): starting from the synthesis of the cytochrome f precursor to the translocation of this precursor through the thylakoid membrane; the processing of the precursor to give a mature protein; the covalent attachment of heme to the apo-protein, and the assembly of cytochrome f with the other subunits to form a functional cytochrome b6f complex. Cytochrome f is required for the assembly of the cytochrome b6f complex because when the petA gene encoding cytochrome f in the chloroplast genome was disrupted, there was no assembled cytochrome b6f complex, and the resulting mutant could not grow photoautotrophically (3,14). Biochemical evidence (4) and the three-dimensional structure (5-8) revealed that there are three domains in cytochrome f: a C-terminal transmembrane span and a small and a large domain. Serving as a transmembrane span, the C-terminal hydrophobic region of cytochrome f (from Gln253 to Phe286 in Chlamydomonas reinhardtii)(9-11) anchors the protein to the thylakoid membrane. Deletion of this region gave rise to a soluble form of cytochrome f (12) that was transported to the lumen and was capable of transferring electrons to plastocyanin, but again there was no assembled cytochrome b6f complex (12). Localized in the lumen, the soluble segment has an elongated structure, with a small and a large domain (5-8) (Figure...
A lysine patch was found in cytochrome f (5-7) from higher plants and *C. reinhardtii* and was postulated to be involved in the interaction with plastocyanin (5). Mutations within the large domain generated interference with the electron transfer (13-21), the proton translocation (18), and the maturation of cytochrome f (13,19). When mutants were isolated in which heme attachment was altered in the large domain of cytochrome f, the assembly of the cytochrome b6f complex and the insertion of heme group were disrupted (13). While these results shed light on the functioning of C-terminal transmembrane-span and the large domain of cytochrome f, the role of the small domain remained ambiguous.

The small domain of cytochrome f consists of amino acid residues from Asn172 to Leu228 (9-11), approximately the difference in size between the lumen soluble segment of cytochrome f and the extra-membrane domain of mitochondrial cytochrome c1 (5). In comparing the primary sequences of cytochrome f from other organisms, the small domain of cytochrome f is less conserved than the large domain (4). Only a few amino acid residues within its sequence are identical among all the sequences found. From the crystal structure of cytochrome f (5-8), we can see that the backbone of the small domain is distinguishable from the large domain (Figure 1). Mutations of amino acid residues within the small domain did not reveal any significant difference from the wild type protein *in vivo* (15-17). These analyses raised the question about the role of the small domain of cytochrome f in the biogenesis and functioning of the cytochrome b6f complex. In a comparison of the structure of the cytochrome b6f complex (5-8, 22, 23) with the analogous cytochrome bc1 complex from mitochondria (24-26), Soriano et al (27) speculated that the small domain of cytochrome f might function like the subunit VIII in the cytochrome bc1 complex to provide structural stability to the complex. Recently the crystal structure of cytochrome f from *C. reinhardtii* revealed that
cytochrome f could exist in dimeric form (7), and amino acid residues from the small domain are found to be involved in the H-bond formation in the interface of this dimer. However, the relationship of this dimer to the structure of cytochrome f in vivo is not clear.

We were interested in defining the role of the small domain of cytochrome f in the biogenesis and functioning of cytochrome f and the cytochrome b6f complex. To address this question, we have made a small domain deletion mutant of cytochrome f in C. reinhardtii. The mutant is able to grow photoautotrophically and has an assembled cytochrome b6f complex. Our studies showed that the electron transfer through the cytochrome b6f complex can occur in the mutant but at a slower rate. In addition, the mutant cytochrome b6f complex was not as stable as that in the wild type strain.

EXPERIMENTAL PROCEDURES

Strains and culture conditions—C. reinhardtii wild type (cc-125), cytochrome f small domain deletion mutant (pFSSD) and ΔpetA mutant (14) strains were maintained on TAP plates (28) under dim light (2 µmol of photons m⁻² s⁻¹). For the photoautotrophic growth of strains, the wild type and the pFSSD mutant were grown on the HS plates (28) under medium light (35 µmol of photons m⁻² s⁻¹). Liquid cultures of the wild type strain and the pFSSD mutant were carried out in HS medium bubbled with 3% CO₂ under 35 µmol of photons m⁻² s⁻¹ light intensity. Bacteria Escherichia coli DH5α strain was used for DNA manipulations.

Construction of Cytochrome f small domain deletion mutant—PCR was performed as described previously (20) to make the construct (Figure 2). The following are the
sequences of primers I-IV: Primer I: 5´-CAACTGGAATCCCCTTATAG-3´ (located 50bp upstream of petA gene); Primer II: 5´-CGCTACGTAATAGTGGTTTGA-3´ (the bold codon encodes Tyr171); Primer III: 5´-GCCTACGTAACAAACAACCCTAACGTGG-3´ (the bold codon encodes Thr229); Primer IV: 5´-GTAGGAGCTGCACAGCAGCC-3´ (located 13bp downstream from the petA gene). The underlined sequence is the SnaBI restriction site. The PCR template was the plasmid pJB101ΔH containing the petA gene (14). A 700bp fragment I was obtained by PCR using primer I and primer II. A new SnaBI site was introduced at the 3´-end of the fragment. Fragment II with a length of 220bp was obtained by PCR using primer III and primer IV. A new SnaBI site was also added to the 5´-end of the fragment II. Fragment I was cut with Hind III and cloned into pUC19 plasmid cut with HindIII and HincII to give pUCFI. Fragment II was cloned into pUC19 cut with HincII to give pUCFII. For the pUCFII, we selected the construct with an orientation where the SnaBI site is adjacent to the HindIII site from the pUC19 polylinker region. The correct sequences of the inserts were confirmed by sequencing in an Applied Biosystems DNA sequencer 377. pUCFII was then digested with SnaBI and EcoRI to give a 220bp fragment, which was ligated with pUCFI cut with the same enzyme to give pUCFSD. pUCFSD was digested with HindIII and AflIII. The insertion was cloned into pJB101ΔH plasmid digested with the same restriction enzyme to give pJBFS. In order to make the chloroplast transformation construct pFSSD, the petA gene sequence from pADFI283ST (3) was replaced with small domain deletion petA mutant sequence from pJBFS by digesting both plasmids with BglII and EcoRV. In the pFSSD construct the sequence of petA gene which encodes small domain of cytochrome f from Gln172 to Leu228 was deleted (57 amino acids sequence). An extra codon for valine was added in the petA gene sequence of the small domain deletion mutant between the codon for Tyr171 and the codon for Thr229 that resulted from the addition of a new SnaBI site.
Chloroplast transformation of C. reinhardtii ΔpetA strain—The ΔpetA strain was transformed as described previously (14) using the biolistic particle delivery system (PDS-1000/He, Bio-Rad). 250 ml TAP medium was inoculated with the ΔpetA strain and grown for three days under dim light. A fresh 600 ml TAP medium was inoculated to approximately $1 \times 10^5$ cells/ml. Cells grown to approximately $2 \times 10^6$ cells/ml in the presence of 0.5 mM fluorodeoxyuridine were harvested by centrifugation and re-suspended to a concentration of approximately $1 \times 10^6$ cells/ml. 1 ml of the cell suspension was mixed with an equal volume of (pre-melted and incubated at 42 °C) 0.2% agar in TAP and 0.7 of the mixture was spread onto 2 pre-prepared TAP plates. 4 µg pFSSD DNA was precipitated onto 0.3 mg gold particles, which were used to bombard the cells on the plates. The bombarded cells were transferred to TAP plates containing 150 µg/ml of spectinomycin and incubated under dim light at 25 °C. PCR was performed to select the transformants as described previously by Berthold et al (29).

Southern blotting, Western blotting, and heme staining—C. reinhardtii DNA was prepared by centrifuging cells from 50 ml culture. Cells were re-suspended in 500 µl of CTAB buffer (2% CTAB, 100 mM Tris-Cl, pH 8, 1.4 M NaCl, 20 mM EDTA, 2% β-mercaptoethanol), and incubated at 65 °C for 60 min. The solution was extracted three times with phenol/chloroform/isoamylalcohol (24:24:1 v/v). The DNA was precipitated with 0.7 volumes of isopropanol. After digesting the DNA with EcoRV and HindIII, Southern blot hybridization was carried out as previously described (14).

Prior to electrophoresis for Western blotting, protein samples were prepared according to Berthold et al.(30). Protein samples were separated on SDS-polyacrylamide gel with a 15% resolving /5% stacking gel and transferred to nylon membranes. Western blotting
was carried out following manufacturer’s protocol (ECL Western blotting, Amersham Pharmacia Biotech). Rabbit sera containing polyclonal antibodies generated against spinach cytochrome f, spinach subunit IV, *C. reinhardtii* Rieske FeS protein (generously provided by Dr. C. de Vitry, Institut de Biologie Physico-Chimique, France), and a synthetic peptide conjugate (corresponding to *C. reinhardtii* cytochrome b6, generously provided by Dr. W. Cramer, Purdue University, USA) were used at a 1:10,000 dilution. Heme peroxidase activity was detected with N, N, N', N'-tetramethylbenzidine and H$_2$O$_2$ (31).

**Oxygen evolution measurements**—Rate of oxygen evolution was measured at 25 °C in an Oxygraph System (Hansatech) according to manufacturer’s instructions. *C. reinhardtii* cells were resuspended at a chlorophyll concentration of 10 µg/ ml in HS medium supplemented with 10 mM Na-bicarbonate (14).

**Flash kinetic measurements**—Kinetic measurements were performed on autolysin treated cells to form a homogenous single-cell suspension, which also allows easy access of inhibitors and uncouplers. Autolysin was prepared as described (28). The pellet of *C. reinhardtii* cells was re-suspended with autolysin solution, and incubated at room temperature for 15 min. Cells were then washed twice with HS liquid medium. A home-built single beam kinetic spectrophotometer with microsecond time resolution was used as described previously (14). Flash-induced spectroscopy was done at 25 °C under anaerobic conditions maintained by argon flux. Cells were suspended in HS medium pH 6.8 (28) at a chlorophyll concentration of 30 µg/ml. A short 23% P700-saturating flash, having a duration of 3.5 µs at half peak height, was used to avoid multiple turnovers of the cytochrome b6f complex. Cytochrome f was monitored as ΔA 554-545 nm (14) in the presence of 30 µM FCCP. For the measurement of cytochrome f oxidation, 22 µM
stigmatellin was also added to inhibit the reduction of cytochrome f. Cytochrome b6 was measured as \( \Delta A \) 564-575 nm (15) in the presence of 30 µM HQNO and 30 µM FCCP. The slow phase of the electrochromic band shift was measured as the difference of \( \Delta A \) 515 nm (18) in the absence and presence of 22 µM stigmatellin. The kinetic data were fit as first-order reactions to give the rate constants, which were used to calculate the halftime of the reactions.

RESULTS

Characterization of the cytochrome f small domain deletion mutant—DNA was isolated from the primary transformants, which were able to grow on the TAP plates containing spectinomycin. The colonies giving the correct size of the DNA fragment by PCR were transferred to HS plates and incubated under dim light at 25 °C. Most of the colonies died after 3-4 weeks. Only a few tiny green colonies were found under the microscope. They were re-streaked onto the fresh HS plates and TAP plates. After a few weeks it was confirmed that pFSSD mutant was able to grow photoautotrophically. The growth rate measured in HS liquid medium under 35 µmol of photons m\(^{-2}\) s\(^{-1}\) showed that the pFSSD mutant grew slightly slower than the wild type (Table I). Under higher light intensity, i.e. 160 µmol of photons m\(^{-2}\) s\(^{-1}\), the slower growth in the pFSSD mutant is more noticeable (doubling time: 9.8 h vs. 8 h). Southern blot analysis revealed the presence of the correct insertion in these cells (Figure 3). In comparison with the wild type strain, the smaller band from the pFSSD mutant showed the correct size of deletion (ca. 0.16 kb) from the petA gene sequence. The chlorophyll contents of the wild type and pFSSD mutant are very similar: The chlorophyll (a+b)/cell is \(3.8 \pm 0.5 \times 10^{-15}\) and \(3.2 \pm 3 \times 10^{-15}\) mol/cell, respectively. The chlorophyll a/chlorophyll b ratio is 2.5 and 2.3, respectively. The presence of the mutant polypeptide was then examined by immunoblotting (Figure 4).
The anti-cytochrome f antibodies identified a band of smaller molecular mass (ca. 25 kDa) in the pFSSD mutant, which is approximately the expected molecular weight for cytochrome f after the deletion of the small domain. The antibodies against cytochrome b6, subunit IV, and Rieske FeS protein also identified bands of the same size as the wild type from the pFSSD mutant, which are in contrast to the petA deletion strain where no detection of any of the subunits of the cytochrome b6f complex is observed (Figure 4, lane 2). It is known that the absence of cytochrome f results in a rapid degradation of the cytochrome b6f complex subunits (3). Therefore the presence of other subunits in the pFSSD mutant suggests that the cytochrome b6f complex was assembled. However the level of these proteins in the mutant cells was only 40-30% of the level in the wild type cells, showing that there was less cytochrome b6f complex in the mutant. Detection of peroxidase activity using tetramethylbenzidine and H$_2$O$_2$ also indicated the presence of a new 25 kDa heme containing protein, suggesting that heme had been covalently inserted into the mutant polypeptide of cytochrome f. We conclude from these studies that deletion of the small domain does not affect the translocation of the protein to the lumen, the processing of the precursor protein, the incorporation of the heme group into the polypeptide, and the small domain is not essential for the assembly of the cytochrome b6f complex.

Figure 5 shows the light saturation curves of photosynthesis for the mutant and wild type cells. The quantum yield of O$_2$ evolution as indicated by the initial slope of the curves is approximately the same for both wild type and the pFSSD mutant. However the pFSSD mutant showed a light-saturated rate of total O$_2$ evolution ca. 5 times lower than that in the wild type. These results suggest that the electron transfer between PSII and PSI is limiting in the mutant under high light conditions.
Electron transfer in cytochrome f small domain deletion mutant—To examine the electron transfer properties of the pFSSD mutant, cells were grown in the HS medium until they reached the mid-exponential phase. After centrifugation, the cells were treated with autolysin. We found that after the autolysin treatment the aggregated cells were dispersed into single cells and that the inhibitors and uncouplers, such as HQNO, went into the cells more readily since the cell wall was removed.

It was considered that the deletion of the small domain from cytochrome f might change the redox differential spectrum of the α-band of cytochrome f. To examine this, a time-resolved oxidized-minus-reduced spectrum of cytochrome f was measured within the range of 540 – 575 nm for the wild type and the pFSSD mutant. The absorbance maxima of the α-band of cytochrome f was found unchanged at 554 nm (Figure 6). The general shape of the spectra were identical between the wild type and the pFSSD mutant (Figure 6 and insert). This suggests that the heme environment in the pFSSD mutant is similar to that of the wild type.

Cytochrome f oxidation and reduction were monitored by following the difference between flash-induced absorbance changes at 554 nm and 545 nm. For measuring the oxidation of cytochrome f, stigmatellin was added to inhibit the reduction of cytochrome f and eliminate the interference from the reduction (14). As shown in Table I, Figure 7a and 7b, the half time (t1/2) of the flash-induced oxidation of cytochrome f in the pFSSD mutant was approximately the same as that of the wild type (ca. 100 µs). The same results were obtained by using a 5 ms time scale and a RC=10µs (data not shown). However, the pFSSD mutant had a t1/2 of cytochrome f reduction at 32 ms, approximately 15 times larger than that of the wild type (Table I, Figure 7c and 7d). This in vivo result indicates that the small domain was required for the reduction but not for
the oxidation of cytochrome f. Since we were using a low intensity flash (23% P700-saturating), which would avoid multiple turnovers of the cytochrome b6f complex, the amplitude of the $\Delta A_{554-545 \text{ nm}}$ changes are of similar magnitude in both wild type and the pFSSD mutant. Under a high intensity flash (75% P700-saturating), less photooxidizable cytochrome f in the pFSSD mutant than that in the wild type was found (ca. 2-3 fold less, data not showed), in agreement with the lower levels of the individual cytochrome b6f subunits detected by immunoblotting in the pFSSD mutant.

The kinetics of cytochrome b6 photoreduction was measured by following the difference between flash-induced absorbance changes at 564 nm and 574 nm. HQNO was added to inhibit the oxidation of cytochrome b6 by plastoquinone (Figure 7e and 7f). In contrast to the reduction of cytochrome f, the half time for the reduction of cytochrome b6 was affected in the mutant, which was 8.6 ms, approximately two fold larger than that of the wild type (4.7 ms) (Table I). This rate of cytochrome b6 reduction in the pFSSD mutant was faster than the rate of the reduction of cytochrome f by a factor of 3.7 ($t_{1/2}$ 8.6 ms vs. $t_{1/2}$ 32 ms). In the mutant the amplitude of cytochrome b6 reduction is smaller by a factor of 1.8 than the amplitude of cytochrome f oxidation, while this ratio of amplitudes is close to 1 in the wild type strain. However, under a high intensity flash (75% P700-saturating) both wild type and pFSSD mutant showed a similar ratio (approximately 1:1) between the amplitudes of cytochrome b reduced and cytochrome f oxidized (data not shown).

The slow phase of the carotenoid electrochromic band shift is believed to serve as an indicator for the charge separation across the thylakoid membrane due to the operation of the cytochrome b6f complex. The charge separation would include the electron movement from the $b_L$ heme to the $b_H$ heme in cytochrome b6 and the proton
translocation from the stroma to the plastoquinone reduction site, known as Qr (32, 33). The electrochromic band shift was followed by the absorbance change at 515 nm. The slow phase associated with the electrogenic reactions in the cytochrome b6f complex is shown as the difference of the traces at 515 nm in the absence and presence of stigmatellin, that inhibits the cytochrome b6f electrogenic activities by blocking the plastoquinol oxidation site (Qo). Figure 7g and 7h show that the slow phase of the electrochromic signal in the pFSSD mutant is slower by a factor of 2 as compared to the wild type (Table I). This suggests that the deletion of the small domain reduced the rate of proton translocation since the electron movement between two cytochrome b hemes is probably intact.

The stability of the cytochrome b6f complex—From the Western blotting and heme staining gel (Figure 4) it was obvious that the amount of the cytochrome b6f complex in the pFSSD mutant was markedly reduced to 30-40% of the level found in the wild type strain. This was also confirmed by flash kinetic spectroscopy experiments under a high intensity flash, in which the amplitude of absorbance changes of cytochrome f and cytochrome b in the pFSSD mutant was always 2-3 fold smaller as compared to the wild type (data not shown). The question arose whether the deletion of the small domain from cytochrome f resulted in an unstable cytochrome b6f complex. To examine the stability of the cytochrome b6f complex in the pFSSD mutant with respect to the wild type, the rates of degradation of the cytochrome b6f complex were studied in the presence of chloramphenicol, an inhibitor of protein synthesis in the chloroplast. Aliquots of the cell culture were removed after the addition of chloramphenicol at different times over a 21 h period and Western blotting was performed for cytochrome f, cytochrome b, Rieske subunit, and subunit IV. Figure 8 shows that in the pFSSD mutant the cytochrome b6f complex subunits, initially present in lower amounts (as in Figure 4), undergo a faster
degradation than those in the wild type strain. The half times for the degradation of these subunits are decreased to about 3-6 h (Table II), whereas the half times for the same subunits in the wild type cells were over 20 h. These experiments indicate that the lack of the small domain of cytochrome f destabilizes the cytochrome b6f complex (27), facilitates the degradation of its subunits, and gives rise to a reduced level of the cytochrome b6f complex in the pFSSD mutant (Figure 4, lane 3; Figure 8B, lane 0).

DISCUSSION

The biogenesis of the cytochrome b6f complex—Cytochrome f is synthesized in the chloroplast stroma and is then inserted into the thylakoid membrane with the bulk of the protein being localized in the lumen. Due to the unique structure of cytochrome f, in which the Tyr1 provides the sixth ligand to the heme iron, the processing of cytochrome f and heme attachment are likely to be closely coupled (5-8). Our results with the pFSSD mutant showed no role for the small domain in these processes. How the heme is inserted into cytochrome f in the chloroplast is still an open question (34, 35). If the insertion of the heme group into cytochrome f requires a heme lyase, which was identified in the mitochondria (36), this lyase does not require the small domain of cytochrome f for carrying out its activities. The cytochrome b6f complex is assembled in the pFSSD mutant, and at low and medium light intensity, the mutant strain grows photoautotrophically at a slightly slower rate than the wild type. It is clear from these results that the small domain of cytochrome f is not playing an important role in the biogenesis of cytochrome f and the cytochrome b6f complex.

Under low light condition, where the limiting factor is the photoactivation frequency of photosystem I and II, the rate of oxygen evolution in the pFSSD mutant is
indistinguishable from that of the wild type. However, under higher light intensities, where other limiting factors dominate, the light-saturated rate of $O_2$ evolution is considerably decreased, pointing to an impairment in the linear electron transfer chain. This is probably due to a combined effect of the lower content of cytochrome b6f complex and the slower electron transfer kinetics in the pFSSD mutant.

**Electron transfer through the cytochrome b6f complex**—Electron transfer through the cytochrome b6f complex in the pFSSD mutant takes place in a manner different from that in the wild type. As the oxidation of cytochrome f by plastocyanin is not affected in the intact mutant cell, this indicates that the small domain is not involved in the interaction with plastocyanin. This result is consistent with *in vivo* studies on the site-directed mutants involving Lys188 and 189 in the small domain (15-16). However, observations from experiments with *in vitro* systems yielded different conclusions. Results from the electron transfer experiments carried out *in vitro* (17, 20, 21) and in nebulized cells (16), and from molecular dynamic modeling (37-39) and the solution structure of the complex between cytochrome f and plastocyanin (40, 41) provided evidence that the lysine residues from the small domain do interact with plastocyanin. This discrepancy between *in vivo* and *in vitro* behaviors remains unresolved. Nevertheless, the reduction rate of cytochrome f in the pFSSD mutant is slower than that in the wild type, as are the reduction of cytochrome b6 and the slow phase of the electrochromic band shift. This is similar to the effect observed when the amino acid residues involved in the formation of an internal water chain were altered (18). We noticed that the half time for the reduction of cytochrome b$_L$ is ca. 2 fold larger than that for the slow phase of electrochromic band shift both in the wild type and the pFSSD mutant (Table I). According to the Q cycle model, these two rates should be the same or the rate of the slow phase of electrochromic band shift should be slower than that of the reduction of cytochrome b6. We would not
discuss the relationship of these two rates here because these two reactions are measured under different condition i.e., in the absence and the presence of FCCP and HQNO, respectively. Further studies are under way to understand the deviation of these two reactions. The oxidation of plastoquinol at the Qo site is generally considered to be non-electrogenic (32, 33) while the reduction of plastoquinone at the Qi site is one of the processes contributing to the slow rise of electrochromic band shift. The question is then how proton translocation from the stroma site is affected while the mutation is localized in a region of cytochrome f that is in the lumen. Under the anaerobic conditions in our experiments the plastoquinol/plastoquinone couple and cytochrome b$_{1}$ are in their reduced form before the flash (42) and the only plastoquinone available after the flash is generated from plastoquinol oxidation at Qo site. The cytochrome b6 reduction signal comes from b$_{L}$ (42), which showed that in the pFSSD mutant the half time for the reduction of the b$_{L}$ heme is increased. The slower reduction of b$_{L}$ indicates a slower oxidation of plastoquinol at the Qo site, followed by a slower release of plastoquinone from the Qo site, resulting in a slower proton translocation at the Qi site. But this interpretation is not sufficient to explain the amplitude of the slow phase of the electrochromic band shift in the pFSSD mutant, which is very similar to the one in the wild type (Figure 7, g; h) and is not affected by the 1.8 fold decreased amplitude of cytochrome b reduction (Figure 7, e; f). This is unexpected if the electrogenicity would be only due to an electron movement through the low-potential chain and a proton movement from the stroma to the Qi site. Instead, a smaller amplitude of cytochrome b6 reduction would result, by the same factor, in a decrease both in electron movement through the low-potential chain and in the provision of plastoquinone from the Qo site to the Qi site. However, the additional unexpected electrogenicity could be explained by an additional proton channel driven by the high-potential chain proposed by Joliot and Joliot (43).
We also observed the loss of the concerted reduction of cytochrome f and b6 in the pFSSD mutant since the reduction of cytochrome b6 was much faster than the reduction of cytochrome f. A similar observation was also reported by Ponamarev et al (18) in cytochrome f mutants, and earlier by others in studies of the wild type cytochrome b6f complex (44). We believe this uncoupling might arise from the electron transfer bypassing cytochrome f under certain conditions (45).

The stability of the cytochrome b6f complex—The stability of the cytochrome b6f complex is affected in the pFSSD mutant where the complex is degraded more rapidly than in the wild type strain. This is consistent with the postulation that the small domain may provide structural stability for the complex (27). But unlike subunit VIII in the cytochrome b/c1 complex (46), the cytochrome b6f complex is still assembled and the deletion of the small domain of cytochrome f did not abolish the complex completely. The lower level of the cytochrome b6f complex in the pFSSD mutant is probably due to a faster degradation of this complex since the rates of the synthesis of cytochrome b6 and subunit IV are the same as the wild type even in the absence of cytochrome f (3). The faster turnover of the cytochrome b6f complex may result from faster degradation of its subunits by protease. As has been shown ClpP, an ATP-dependent protease which has been found in the stroma (47), is able to degrade the assembled form of the cytochrome b6f complex (48).

The cytochrome b6f complex may exist as a dimer in vivo (49-51). Interestingly, cytochrome f can also form dimeric associations in the crystal (7), in which amino acid residues from the small domain of one monomer make close contact with residues from another monomer. In either case of cytochrome f forming a dimeric association in a
cytochrome b6f complex dimer or interacting with other subunits of the complex, the deletion of the small domain may have eliminated part of the interaction between two cytochrome f monomers in the dimeric form of the cytochrome b6f complex or between cytochrome f and other subunits of the complex, resulting in a complex which may be “looser” and more prone to the degradation by protease.

In summary, our studies on a small domain deletion mutant of cytochrome f have shown that the small domain is not required for the biogenesis of cytochrome f and the cytochrome b6f complex. In the small domain deletion mutant of cytochrome f, electron transfer through the cytochrome b6f complex occurs but at a slower rate than that in the wild type strain. The small domain of cytochrome f is also important in stabilizing the cytochrome b6f complex.

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REFERENCES

FOOTNOTES

1 The abbreviations used are:

CTAB: Hexadecyltrimethyl-ammonium bromide; FCCP: Carbonylcyanide p-trifluoromethoxy-phenylhydrazone; FeS: Iron sulfur redox center; HQNO: 2-n-Heptyl-4-hydroxy-quinoline-N-oxide; PCR: Polymerase chain reaction; PSI: Photosystem I; PSII: Photosystem II; Qi and Qo sites: Plastoquinone reduction and oxidation sites in the cytochrome b6f complex, respectively.
FIGURE LEGENDS

Fig. 1. Three dimensional structure of cytochrome f. The structure is derived from the coordinates of *C. reinhardtii* cytochrome f (7) and was drawn using MOLSCRIPT (52). The small domain and large domain are indicated.

Fig. 2. Details of the construction for the small domain deletion mutant of cytochrome f. The cytochrome f coding region is shown as a box, with the region encoding the presequence and mature sequence marked P and Mature, respectively. The location of the restriction sites used for the construction of pFSSD (see Experimental Procedures) are shown. A: AflII; B: BamHI; E: EcoRV; H: HindIII; S: SnaBI. The sequence between Tyr171 and Thr229 is deleted in the pFSSD construct. The locations of the primers used for mutagenesis are shown. Primer I and II are used for PCR Fragment I. Primer III and Primer IV are used for PCR Fragment II.

Fig. 3. Southern blot hybridization of *C. reinhardtii* DNAs. Total DNA was isolated from wild type (lane 1), ΔpetA mutant (lane 2), and two strains of the pFSSD mutant (lane 3 and 4), and was digested by EcoRV and HindIII. Southern blot hybridization was performed using a probe of about 600 bp, which was prepared by digesting the petA gene with AccI and Hind III.

Fig. 4. Immunoblot analysis (panel A) and heme staining (panel B) of the cytochrome b6f complex subunits from wild type and the pFSSD mutant of *C. reinhardtii*. Total cellular protein from *C. reinhardtii* wild type (lane 1), ΔpetA (lane 2), and pFSSD (lane 3) was isolated. After SDS-PAGE, immunoblots against cytochrome f,
cytochrome b6, subunit IV, and Rieske FeS protein were performed. Heme staining was done using tetramethylbenzidine and H$_2$O$_2$. All samples were loaded with an amount equivalent to 10 µg chlorophyll/lane.

**FIG. 5.** **Light saturation curves of net photosynthesis.** *C. reinhardtii* cells were grown under 35 µmol of photons m$^{-2}$ s$^{-1}$ and were collected after they reached the early exponential phase. Curves for wild type (square) and the pFSSD mutant (triangle) are shown. The same sample was used and it was illuminated for 2.5 min at each increasing light intensity.

**FIG. 6.** **Flash-induced absorbance changes of cytochrome f in the 540-575 nm region.** A single 75% P700-saturating flash was given to the wild type (insert) and pFSSD (circles) cells, which were suspended in HS medium supplemented with 30 µM of FCCP. The absorbance changes at different wavelengths were recorded 3 ms after the flash.

**FIG. 7.** **Kinetics of electron transfer and charge separation through the cytochrome b6f complex.** The flash-induced absorbance changes of the cytochrome b6f complex in wild type (panel A) and the pFSSD mutant (panel B) cells are shown. A single 23% P700-saturating flash was given at the time zero. Traces for cytochrome f in the presence (a, b) and the absence (c, d) of 22 µM stigmatellin; for cytochrome b6 in the presence of HQNO (e, f); and for ΔA of slow electrochromic band shift (g, h) are shown. All traces are results from 160 to 320 averages. Instrumental time constant (RC) was 50 µs, 200 µs, and 20 µs for traces (a-d), (e, f), and (g, h), respectively.

**FIG. 8.** **The time course of the cytochrome b6f complex degradation.** Total cellular protein from *C. reinhardtii* wild type (panel A) and the pFSSD mutant (panel B) cells
were collected at various time points after the addition of chloramphenicol. Cultures were grown under 35 µmol of photons m\(^{-2}\) s\(^{-1}\) light intensity bubbled with 3% CO\(_2\) at 20 °C. Chloramphenicol was added at time zero (100 µg/ml) after the cultures reached the mid-exponential phase. Western blotting was done using antibodies against cytochrome b6, Rieske FeS protein, subunit IV, and cytochrome f, respectively, to estimate the amount of the proteins in the cells. The amount of protein sample loaded in each lane was equivalent to 10 µg of chlorophyll.
### TABLE I

*Comparison of physiological and kinetic parameters between wild type and the pFSSD mutant*

The range represents the maximum variation of data collected from different experiments with different cultures.

<table>
<thead>
<tr>
<th></th>
<th>Doubling time (hours)$^a$</th>
<th>Oxygen evolution (nmol O$_2$ µmol Chl$^{-1}$ min$^{-1}$)$^b$</th>
<th>Cytochrome f oxidation $t_{1/2}$ (ms)</th>
<th>Cytochrome f reduction $t_{1/2}$ (ms)</th>
<th>Cytochrome b6 reduction $t_{1/2}$ (ms)</th>
<th>Slow Δψ 515nm $t_{1/2}$ (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>12.4 ± 0.3</td>
<td>2222 ± 81</td>
<td>0.14 ± 0.03</td>
<td>2.1 ± 0.3</td>
<td>4.7 ± 0.4</td>
<td>2.7 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>(4)$^c$</td>
<td>(3)</td>
<td>(5)</td>
<td>(5)</td>
<td>(8)</td>
<td>(6)</td>
</tr>
<tr>
<td>pFSSD</td>
<td>13.9 ± 0.3</td>
<td>400 ± 2</td>
<td>0.10 ± 0.04</td>
<td>32 ± 5</td>
<td>8.6 ± 2.9</td>
<td>5.5 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>(4)</td>
<td>(2)</td>
<td>(8)</td>
<td>(10)</td>
<td>(9)</td>
<td>(6)</td>
</tr>
</tbody>
</table>

*a: Cells were grown in the HS medium under 35 µmol of photons m$^{-2}$s$^{-1}$, bubbling with 3% CO$_2$*  
*b: Net photosynthesis. Incident intensity 2530 µmol of photons m$^{-2}$s$^{-1}$ . Experiments at 25 °C*  
*c: The number of total trials is given in brackets for each case.*
The rates of the degradation of cytochrome b6f complex were measured using immunoblotting as shown in Figure 8. The intensities of the individual bands were quantified by NIH image software. The range represents the variation of data collected from two experiments.

<table>
<thead>
<tr>
<th></th>
<th>t1/2 (h)</th>
<th>t1/2 (h)</th>
<th>t1/2 (h)</th>
<th>t1/2 (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome b6</td>
<td>Wild type</td>
<td>&gt;21</td>
<td>&gt;21</td>
<td>20 ± 8</td>
</tr>
<tr>
<td></td>
<td>pFSSD</td>
<td>3.5 ± 0.2</td>
<td>4.0 ± 0.1</td>
<td>5.8 ± 1.9</td>
</tr>
<tr>
<td>Rieske</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Subunit IV</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytochrome f</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Flash-induced absorbance change (x 10^3)

\[ \Delta A_{515nm} \]

- \[ Cytf \] (A)
- \[ Cytf \] (B)
- \[ Cytf \] (C)
- \[ Cytf \] (D)
- \[ Cytf6 \] (E)
- \[ Cytf6 \] (F)
- \[ \Delta A_{515nm} \] (G)
- \[ \Delta A_{515nm} \] (H)

Time (ms)
A
0 3 6 9 12 15 18 21

Cytb6
Rieske
SubIV
Cytf

B
0 3 6 9 12 15 18 21 (h)

Cytb6
Rieske
SubIV
Cytf
Electron transfer and stability of the cytochrome b6f complex in a small domain deletion mutant of cytochrome f
Xiao-Song Gong, Susana Chung and Javier G. Fernández-Velasco

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