The Plant Cell, Vol. 11, 2031–2044, October 1999, www.plantcell.org © 1999 American Society of Plant Physiologists

Analysis of the Nucleus-Encoded and Chloroplast-Targeted Rieske Protein by Classic and Site-Directed Mutagenesis of Chlamydomonas

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Three mutants of the alga *Chlamydomonas reinhardtii* affected in the nuclear *PETC* gene encoding the Rieske iron–sulfur protein 2Fe–2S subunit of the chloroplast cytochrome b_6f complex have been characterized. One has a stable deletion that eliminates the protein; two others carry substitutions Y87D and W163R that result in low accumulation of the protein. Attenuated expression of the stromal protease ClpP increases accumulation and assembly into b_6f complexes of the Y87D and W163R mutant Rieske proteins in quantities sufficient for analysis. Electron-transfer kinetics of these complexes were 10- to 20-fold slower than those for the wild type. The deletion mutant was used as a recipient for site-directed mutant *petC* alleles. Six glycine residues were replaced by alanine residues (6G6A) in the flexible hinge that is critical for domain movement; substitutions were created near the 2Fe–2S cluster (S128 and W163); and seven C-terminal residues were deleted (G171och). Although the *6G6A* and *G171och* mutations affect highly conserved segments in the chloroplast Rieske protein, photosynthesis in the mutants was similar to that of the wild type. These results establish the basis for mutational analysis of the nuclear-encoded and chloroplast-targeted Rieske protein of photosynthesis.

INTRODUCTION

Photosynthesis, organelle biogenesis, and many other processes in plant cells involve the coordinated activity of nuclear and plastid-encoded proteins. Elucidation of these processes will require multiple approaches, including nuclear transformation with modified genes. Genes have been widely introduced into plant nuclei; however, site-directed mutagenesis of plant nuclear genes has not been reported (Bevan et al., 1999). Homologous insertion and replacement events are extremely rare in both plant and algal nuclear genomes.

DNA introduced into the green alga *Chlamydomonas reinhardtii* cells by particle bombardment or other methods integrates largely randomly into the nuclear genome and permits the inactivation and tagging of selected genes (reviewed in Kindle, 1998). Recently, site-directed mutagenesis and nuclear transformation have been used to introduce modified genes for the γ subunit of coupling factor 1 (Ross et al., 1995) and the PsaF subunit of photosystem I (PSI; Hippler et al., 1998) into Chlamydomonas mutants carrying null mutations of these genes. Chlamydomonas cells possess the photosynthetic apparatus and fundamental features of plant cells but are facultative phototrophs, synthesizing the photosynthetic apparatus under heterotrophic as well as phototrophic growth conditions. They serve as amenable and unique models for mutational studies of plant cell functions involving nuclear genes. We exploited this potential for studies of the nuclear-encoded Rieske iron–sulfur protein of the chloroplast cytochrome b_6f complex, a transmembrane proton pump of the photosynthetic electron transfer chain.

The high midpoint potential (E_m of $\sim$+300 mV) Rieske 2Fe–2S protein plays essential roles in photosynthetic electron transport, redox sensing, and signal transduction in chloroplasts (reviewed in Keren and Ohad, 1998; Wollman, 1998). The cytochrome b_6f complex transfers electrons from photosystem II (PSII) to PSI by plastoquinol oxidation and reduction of plastocyanin and contributes to translocation of protons across the thylakoid membrane. The Rieske protein catalyzes the oxidation of plastoquinol at the Q_o site close to the lumenal membrane face, leading to bifurcated electron transfer along both a high-potential chain (via the Rieske protein and cytochrome *f* to plastocyanin) and a low-potential chain (via the hemes b_l and b_h, allowing electron transfer across the membrane to a quinone at the Q_i site near the

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stromal face of the thylakoid membrane). Related cytochrome *bc* complexes are part of mitochondrial and bacterial respiratory chains. High-resolution structures have been determined for the soluble domains of the bovine mitochondrial (Iwata et al., 1996) and spinach chloroplast (Carrell et al., 1997) Rieske proteins and show generally similar organization. Recent x-ray crystal structures of these complexes have shown that the Rieske protein comprises a soluble domain tethered by a flexible region to a transmembrane anchor (Xia et al., 1997; Kim et al., 1998; Zhang et al., 1998). The soluble domain occupied different positions in bc_1 complexes that were cocrystallized with inhibitors, which suggests a unique domain movement during catalysis (Kim et al., 1998; Zhang et al., 1998).

The chloroplast Rieske protein is encoded by the nuclear *PETC* gene and is imported into the chloroplast via a cleavable presequence; the soluble domain is translocated across the thylakoid membrane and into the chloroplast lumen via residues within the N-terminal hydrophobic domain of the mature protein (de Vitry, 1994; Madueño et al., 1994). The assembly of the 2Fe–2S cluster and of the Rieske protein into the b_6f complex, as well as the role of the protein in electron transfer and signal transduction, are the subjects of ongoing research. The Chlamydomonas *PETC* gene has been cloned (de Vitry, 1994). This unicellular alga is readily amenable to pulse labeling, has a rapid growth rate (generation time ~8 hr), and is a facultative phototroph; its haplobiontic cell cycle allows a variety of genetic studies; and transformation of the nucleus and chloroplast genome is easily performed (Rochaix et al., 1998). The cytochrome b_6f complex has been purified (Pierre et al., 1995), and measurements of rapid electron transfer reactions in vivo (reviewed in Joliot et al., 1998) are possible. A Chlamydomonas Rieske-deficient mutant, *ac21*, has long been available (Levine and Smillie, 1962) and assembles the remaining subunits of the b_6f complex (Lemaire et al., 1986); however, this mutant reverts. Additional, more stable Rieske-deficient mutants *788* (*petC-Δ1*) and *794* are described in the present work. The use of a Chlamydomonas mutant deficient in ClpP protease (W. Majeran, F.-A. Wollman, and O. Vallon, manuscript in preparation) allowed the overexpression of the defective ac21 (*petC-W163R*) and 794 (*petC-Y87D*) Rieske proteins in quantities that permitted biophysical analysis.

Rieske proteins of bc_1 complexes have been studied extensively by using mutagenesis (reviewed in Graham et al., 1993; Kallas, 1994) and suppressor analyses. Residues with roles in cluster binding, midpoint potential determination (Gatti et al., 1989; Liebl et al., 1997; Denke et al., 1998; Schroter et al., 1998), and domain interactions within the bc_1 complex Rieske proteins and with neighboring subunits have been defined (Brasseur et al., 1997; Saribas et al., 1998). Site-directed mutagenesis of b_6f subunits of cyanobacteria is also possible (Wu et al., 1995); however, only nonlethal mutants can be propagated because the b_6f complex is required for both photosynthetic and heterotrophic growth (reviewed in Kallas, 1994). Mutational investigations of chloroplast Rieske proteins have not been attempted. For initial study (de Vitry et al., 1998), we chose substitutions of the conserved S128 residue, which is involved in cluster assembly and redox potential determination, and of conserved aromatic residue W163, which may play a role in stability and electron transport. We also created mutants in the flexible neck region implicated in the Rieske protein movement and in the unique conserved 12- to 14-residue C-terminal extension of b_6f Rieske proteins.

RESULTS

Mutants Deficient in the Rieske Protein Accumulate the Other Cytochrome b_6f Complex Subunits, with an Apparently Modified Cytochrome b_6

Mutant *ac21*, obtained by UV mutagenesis (Levine and Smillie, 1962), was identified as being deficient in the Rieske protein (Lemaire et al., 1986). Mutants *788* and *794* also were obtained by UV mutagenesis and were identified in a collection of b_6f mutants kindly provided by S. Merchant (University of California, Los Angeles). Heme staining of the cells shown in Figure 1A demonstrates that *ac21*, *788*, and *794* accumulated cytochromes *f* and b_6 as heme binding proteins. The results of the immunodetection analysis shown in Figure 1B indicate that the b_6f subunits, except the Rieske protein, accumulated to ~60% of wild-type amounts. Mutants deficient in the Rieske protein had a cytochrome b_6 of higher apparent molecular mass than did the wild type when the proteins were electrophoresed (Figures 1A and 1B).

Immunodetection of cell extracts revealed the complete absence of the Rieske protein in the *788* mutant and variable but low accumulation in the *794* and *ac21* mutants, typically at ~1 and 4% of the wild-type amount, respectively (in early exponential-phase cultures of $\sim 10^6$ cells/mL). The apparent molecular mass of the Rieske protein when electrophoresed was higher in the *794* mutant and lower in the *ac21* mutant than in the wild type. Analysis of polypeptides synthesized on cytoplasmic ribosomes and inserted into thylakoid membranes during 7 min of pulse labeling showed (1) insertion of the Rieske protein into thylakoids in the wild type and the *ac21* mutant, (2) a lower apparent molecular mass of the Rieske protein in the *ac21* mutant (as shown in Figure 1B), and (3) less intense labeling of the Rieske protein in the *ac21* mutant compared with that in the wild-type cells (Figure 1C).

Localization of the Mutations in the Nuclear *PETC* Gene Encoding the Chloroplast Rieske Protein

Genetic analyses are detailed in Table 1. The absence of recombination and complementation indicated that the *788*, *794*, and *ac21* mutants were affected in the same gene.

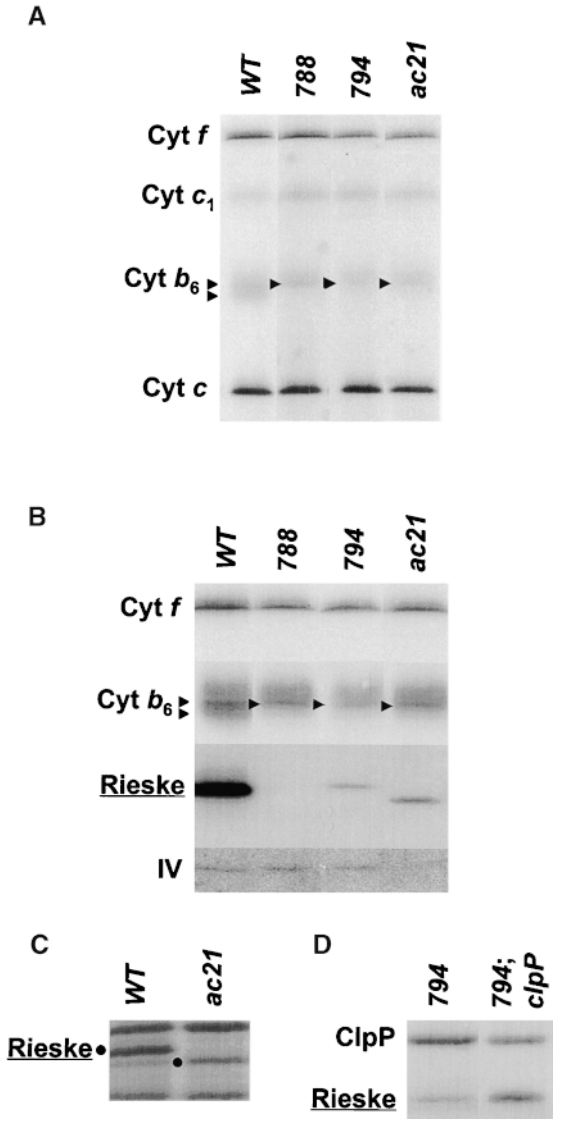

Figure 1. Subunits of the Cytochrome b_6f Complex in Rieske Protein–Deficient Mutants.

(A) Heme staining of Chlamydomonas wild-type and mutant cell extracts separated by SDS-PAGE.
(B) Immunodetection (cell extracts).
(C) The Rieske protein of the wild type and *ac21* mutant seen after 7 min of ^{14}C acetate pulse-labeling in the presence of chloramphenicol (membrane extracts).
(D) Immunodetection (cell extracts) of ClpP protease and Rieske protein in the single mutant *794* (named *petC-Y87D* after molecular characterization) and the double mutant *petC-Y87D clpP-AUU* (*794* mutant attenuated in stromal protease ClpP expression).
Cyt, cytochrome; IV, subunit IV; *WT*, wild type.

PETC transcript analysis is shown in Figure 2. The *PETC* transcript was undetectable in the *788* mutant, equaled the wild-type quantity in the *794* mutant, and overaccumulated in the *ac21* mutant. Complementation with a 4.5-kb genomic *PETC* DNA fragment restored phototrophy and the wild-type quantity of the *PETC* transcript and indicated that the *788* and *794* mutants are directly affected in the *PETC* gene (de Vitry et al., 1998). *PETC* complementation was not performed with *ac21* mutant because of a high frequency of reversion events. Revertants of *788* were not detected, whereas reversion occurred in approximately one per 10^7 *ac21* cells.

Sequencing of polymerase chain reaction (PCR) products identified lesions in the *PETC* gene that cause (1) a frameshift (TG deletion in codon 7 of the mature protein) leading to a truncated Rieske protein of 52 residues in the *788* mutant (*petC-Δ1*) with a second, probably neutral mutation in intron 3 (G784A; numbered relative to the initiator ATG codon of the wild-type preprotein); (2) the substitution Y87D (TAC→GAC) in the mature Rieske protein of mutant *794*; and (3) the substitution W163R (TGG→CGG) in the mature Rieske protein of mutant *ac21.*

Dimeric Assembly of Cytochrome b_6f Complex in Mutants Deficient in the Rieske Protein

Cytochrome b_6f complexes were purified from exponentially growing cells of the Rieske-deficient mutant and wild type by a modification of the Hecameg solubilization procedure described in Pierre et al. (1995). Figure 3 shows immunoblot analysis of the distribution of the cytochrome *f* and Rieske proteins upon sucrose gradient centrifugation. The distribution of these proteins peaked in fractions of similar sucrose density, at a position known to be that of the dimeric form of the wild-type b_6f complex (Breyton et al., 1997). In the *ac21*

Table 1. Complementation, Reversion, and Recombination Analyses of the Rieske-Deficient Mutants

Mutant	*ac21*	*788*	*794*
ac21	10^{-7}[a]	0/74[b]	0/81[b]
788	—[c]	$<10^{-9}$[a]	0/79[b]
794	—[c]	—[c]	$<2 \times 10^{-8}$[a]

[a] Reversion results are shown on the diagonal.
[b] Recombination results are shown above the diagonal (top and right). The growth of progeny from zygotes was assessed on minimal medium versus rich Tris-acetate-phosphate medium.
[c] Complementation results are shown under the diagonal (bottom and left). A dash indicates that the fluorescence kinetics of zygotes during dark-to-light transition displayed curves corresponding to a mutant phenotype and failed to complement.

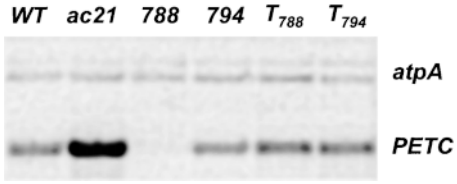

Figure 2. *petC* Transcript Analysis of Rieske Protein–Deficient Mutants.

T_{788} and T_{794} are the *788* and *794* mutants transformed with the wild-type *PETC* gene encoding the Rieske protein. The *atpA* transcript was used as a control. *WT*, wild type.

(*petC-W163R*) mutant, some of the Rieske protein assembled into b_6f complexes (Figure 3). The cytochrome *f*, cytochrome b_6, subunit IV, PetG, PetL, and PetM subunits comigrated in the gradients from the *788* (*petC-Δ1*) and *ac21* (*petC-W163R*) mutants, indicative of their assembly into complexes in these mutants (data not shown).

Stabilization of Mutant Rieske Proteins by Attenuated Expression of the Stromal Protease ClpP

When a mutation affects the properties of a protein complex without compromising its stability, this provides valuable material for functional analysis. The other subunits of the Chlamydomonas b_6f complex assemble even in the absence of the Rieske protein. However, some mutations in the Rieske protein, such as *petC-Y87D* and *petC-W163R*, allowed only very low accumulation of the Rieske protein. A chloroplast mutant that attenuates the expression of the stromal protease ClpP has been created by site-directed mutagenesis of the *CLPP* gene, resulting in a fourfold decrease in the chloroplast content of ClpP (W. Majeran, F.-A. Wollman, and O. Vallon, manuscript in preparation). Because nuclear mutations have biparental heredity, whereas chloroplast mutations are transmitted uniparentally by the mating type (+), we attempted to improve the stability of the mutant Rieske protein by crossing the nuclear mutants *petC-Y87D* and *petC-W163R* of mating type (−) with the chloroplast *clpP-AUU* mutant of mating type (+). In the resulting double mutants, *petC-Y87D clpP-AUU* and *petC-W163R clpP-AUU*, the accumulation of the Rieske protein could be increased in early exponential-phase cultures to ~8% (Figure 1D) and 40% (W. Majeran, F.-A. Wollman, and O. Vallon, manuscript in preparation) of the wild-type amount, respectively. We observed an increase in the growth rate of the double mutant *petC-W163R clpP-AUU* but no growth of the *petC-Y87D* or *petC-Y87D clpP-AUU* mutants under phototrophic conditions.

Electron Transfer Is Blocked in the Deletion Mutant *petC-Δ1* and Considerably Slowed in the Substitution Mutants *petC-Y87D* and *petC-W163R*

Figure 4 shows the patterns of fluorescence induction at room temperature of dark-adapted wild-type and mutant cells when illuminated continuously. The addition of 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea (DCMU) prevents reoxidation of the primary PSII quinone acceptor and leads to maximal fluorescence yield F_{max}. In the absence of DCMU, the initial fluorescence value defines F_0, and the steady state fluorescence value defines F_{stat}. The ratio $(F_{max}-F_{stat})/(F_{max}-F_0)$ is a relative measure of the linear electron flow (LEF): the smaller the difference, the lower the reoxidation rate of the PSII acceptor by the downstream electron transport chain. Table 2 summarizes the LEF results. The *petC-Δ1* mutant clearly was completely blocked in PSII reoxidation and therefore in photosynthetic LEF. The *petC-Y87D* (data not

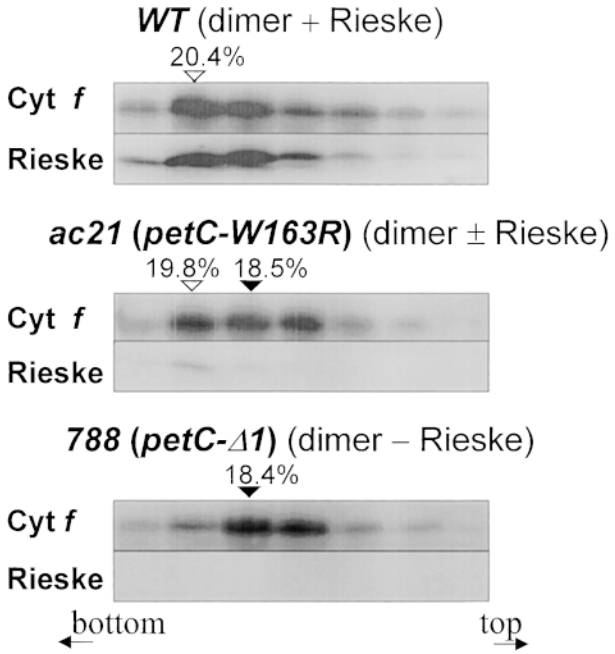

Figure 3. Dimeric State of the Cytochrome b_6f Complex in Rieske Protein–Deficient Mutants.

The cytochrome b_6f complex was purified from thylakoid membranes by using the Hecameg solubilization technique (see Results). The supernatant was centrifuged on a 10 to 30% sucrose gradient. Fractions from the gradient were analyzed by SDS-PAGE, and cytochrome *f* and the Rieske protein were immunodetected. The open and filled arrowheads indicate the sucrose concentration at the peak of cytochrome b_6f complex in the gradient with and without Rieske protein, respectively. The sucrose concentration of the fraction is given above the arrowhead as a percentage. Cyt, cytochrome; *WT*, wild type; (+), with; (±), with or without; (−), without.

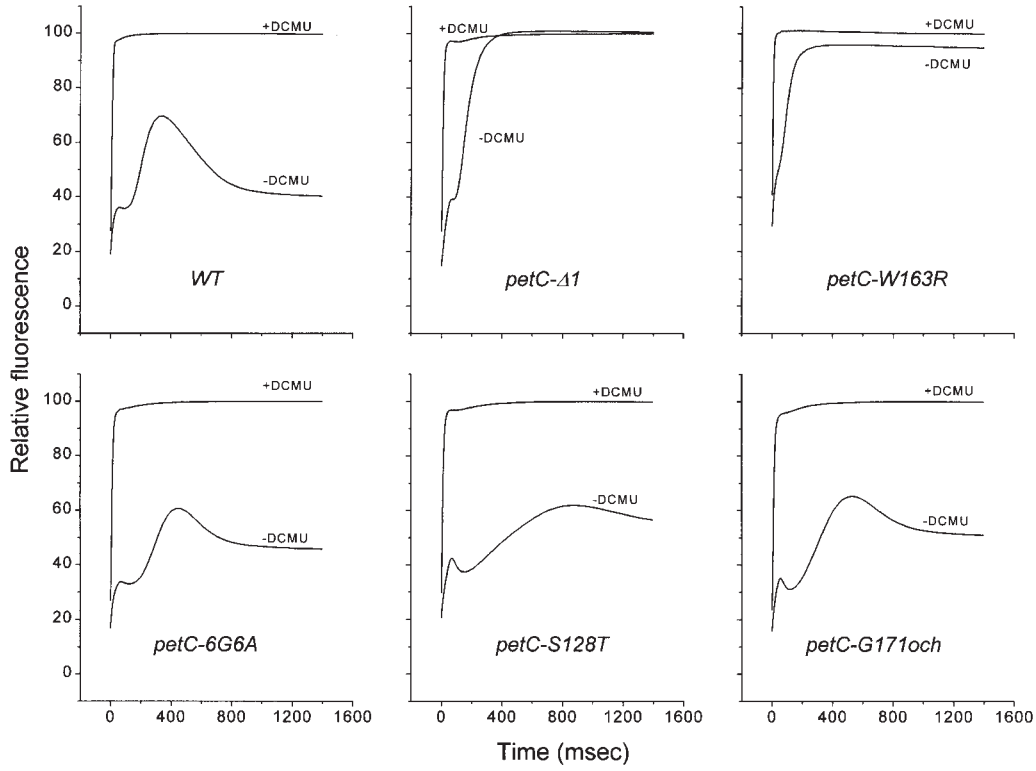

Figure 4. Fluorescence Induction Transients of Dark-Adapted Cells from *WT*, *petC-Δ*1, *petC-W163R*, and Site-Directed Rieske Mutants in the Presence or Absence of 10 μM DCMU.

Maximal levels of fluorescence (F_{max}) were normalized to 100%.

shown) and *petC-Y87D clpP-AUU* (Table 2) mutants also seemed to be blocked in linear electron transfer under these conditions. The fluorescence pattern of *petC-W163R* (Figure 4) and *petC-W163R clpP-AUU* (Table 2) showed little difference and significant difference, respectively, between F_{max} and F_{stat}, which is indicative of increased efficiency of linear LEF when ClpP is attenuated.

Table 2 summarizes b_6f kinetics in vivo. The half-time of the slow rise of the electrochromic shift of carotenoids at 515 nm was induced by changes in the transmembrane potential during charge transfer through the b_6f complex. The permanent electrochemical potential of the living algae is abolished by addition of the uncoupler carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP). In agreement with previous reports (Finazzi et al., 1997), the wild-type cells showed a half-time of 1.2 to 2.4 msec under these conditions. The half-time of cytochrome *f* reduction was 1.8 to 2.7 msec for the wild type. In contrast, in the *petC-Δ*1 mutant, the slow rise of the carotenoid band shift was absent. Cytochrome *f* became oxidized by plastocyanin, but its rereduction was 10^4 times slower than in the wild type (Finazzi et al., 1997). This value is similar to that observed in a mutant that accumulates a soluble cytochrome *f* and lacks the remainder of the b_6f complex (Kuras et al., 1995), which indicates that this reduction is not mediated by the Q_o site.

The quantity of Rieske protein–containing b_6f complexes in the mutants *petC-Y87D* and *petC-W163R* was far below the wild-type amount. Charge separation in PSI induced by an actinic flash therefore will induce oxidation of more plastocyanin molecules than functional b_6f complexes. This induces multiple turnover of the few active complexes and results in increased half-times of the slow rise of the carotenoid band shift and of cytochrome *f* reduction. To obtain accurate half-time values for single turnovers of the b_6f complex, we used the double mutants *petC-Y87D clpP-AUU* and *petC-W163R clpP-AUU.* These accumulated more Rieske protein than did the single mutants bearing the *petC* mutations alone. Furthermore, the intensity of the actinic light was lowered until further attenuation did not lead to additional acceleration of the reaction kinetics. Time-resolved spectroscopic analysis of the *petC-W163R clpP-AUU* mutant is shown in Figures 5A and 5B. Real half-time values for electron transfer in b_6f complexes of *petC-W163R* and *petC-Y87D* were 10- to 20-fold lower than wild-type values

Table 2. Functional Properties of the Rieske Mutants

Strain	Phototrophic Growth[a]	LEF[b]	Phase b[c] $t_{1/2}$ msec	Cyt *f* Reduction[d] $t_{1/2}$ msec
Wild type	+	0.7	1.2 to 2.4	1.8 to 2.7
petC-Δ1	−	0	undetected	10,000
petC-6G6A	+	0.7	2.6	WT
petC-Y87D;clpP-AUU	−	0	40	50
petC-S128T	+	0.5	2.0	WT
petC-W163R;clpP-AUU	+	0.3	25	30
petC-G171och	+	0.6	2.0	WT

[a] Phototrophic (+); nonphototrophic (−).

[b] LEF was estimated from fluorescence induction kinetics as $(F_{max}-F_{stat})/(F_{max}-F_o)$; F_{max}, maximum level of fluorescence with DCMU; F_{stat}, level of fluorescence after 1.4 sec without DCMU; F_o, initial fluorescence level without DCMU.

[c] Phase b of the light-induced, electrochromic shift of carotenoid absorbance measured at 515 nm represents the transmembrane charge transfer in cytochrome (Cyt) b_6f complex. Measurements were made with nonsaturating flashes (3 to 20% of saturation) in the presence of 1 μM FCCP. For the wild type, a range of typical values is shown. $t_{1/2}$ msec, half-time in milliseconds.

[d] Time-resolved rereduction of cytochrome *f*. WT denotes that half-time was in the range of typical values of the wild type.

(Table 2). Kinetics of the electrochromic shift and of cytochrome *f* reduction were proportionally affected in both mutants. These data indicate a perturbation in the concerted electron transfer from the quinol at the Q_o site to the Rieske protein.

Strategy for Site-Directed Mutagenesis of the Nuclear-Encoded Rieske Protein

Mutations created in the chloroplast Rieske protein are described in Table 3 (see Methods) and shown in a model of the protein in Figure 6. These replace glycine residues in the flexible hinge region implicated in domain movement (6G6A), create substitutions of S128 (S128A, S128C, and S128T) or of the aromatic residue W163 (W163A) near the 2Fe–2S cluster, or delete residues from the C terminus (G171och).

Mutant *788* (*petC*-Δ1) was used as the recipient for site-directed mutations. Mutant *petC* alleles contained a BamHI restriction site tag in intron 1 and a second, specific restriction tag associated with each directed mutation (Table 3). Mutant *i1Bam* is a control containing only the BamHI restriction site tag in intron 1. For our initial study, we focused on Rieske protein mutations that could support photosynthesis, selecting these by their ability to grow phototrophically on minimal medium. Phototrophic transformants were recovered at a frequency of ~1 to 10 per 10^8 recipient cells after transformation with plasmids carrying the *i1Bam*, *6G6A*, *S128T*, and *G171och petC* alleles.

To confirm that phototrophic colonies arose from integration of modified *petC* alleles and not from reversion, recombination, or gene conversion events that might restore a wild-type *PETC* gene, we performed allele-specific PCR and restriction tests (Figure 7). Under stringent conditions, the primers 788ΔTG and C5aFS amplified only the defective *788* *petC* allele and not the wild type or introduced *petC* alleles (Figure 7A). These primers generated the expected 1242-bp PCR products from mutant *788* and from *788* transformed with the *6G6A*, *S128T*, and *G171och petC* alleles but not from wild-type Chlamydomonas (Figure 7C). Mutant *788* and these transformants therefore retained copies of the defective *788 petC* allele. Primers WT107TG and C5aFS amplify *petC* alleles that are wild type in exon 1 (Figure 7B). Accordingly, these primers generated the expected 1243-bp PCR product from wild-type Chlamydomonas and from mutant *788* transformed with the mutant *petC* alleles but did not amplify DNA from the original *788* mutant (Figure 7D). To confirm that these transformants contained the mutant *petC* genes, PCR products from the WT107TG/C5aFS primer set were digested with restriction enzymes specific for the mutant *6G6A*, *S128T*, and *G171och petC* alleles. The nearly complete digestion of these products and generation of expected restriction fragments (Figure 7E) confirm that restoration of phototrophy to the *788* (*petC-Δ1*) mutant resulted from transformation with specific mutant *petC* alleles.

Subunits of the Cytochrome b_6f Complex and Restoration of the Expected Full-Length or Truncated Rieske Proteins in Transformants of Mutant *788* (*petC-Δ1*)

Comparable amounts of cytochromes *f* and b_6 were found in the wild type and transformants (Figure 8A). Furthermore, as shown in Figure 1A and in lane 1 of Figure 8A, the wild type typically displays two diffuse 3,3′,5,5′-tetramethylbenzidine–stained bands for cytochrome b_6. The lower band was undetectable in the Rieske deletion mutant *788* but was restored in the transformants. Figure 8B shows restoration of the Rieske protein to mutant *788* (*petC-Δ1*) transformed with mutant *petC* alleles. The G171och Rieske protein migrated

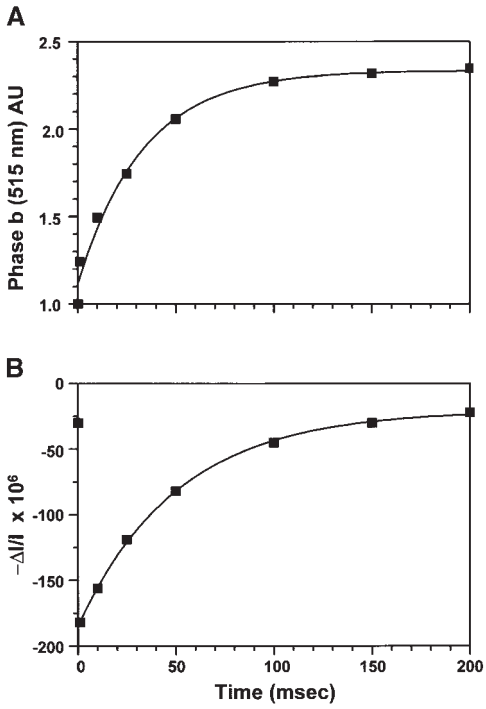

Figure 5. Time-Resolved Spectroscopic Analysis of the Double Mutant *petC-W163R clpP-AUU*.

petC-W163R was named *ac21* before molecular characterization. *clpP-AUU* mutants are attenuated in stromal protease ClpP expression.
(A) Kinetics of the slow electrochromic phase at 515 nm indicative of charge transfer across the membrane with nonsaturating actinic flashes (10% saturation). AU, arbitrary units.
(B) Time-resolved redox changes of cytochrome *f*. $-\Delta I/I$ is the difference between the light transmitted through the measuring and the reference cuvettes divided by the light transmitted through the reference cuvette (Joliot et al., 1998).

at a lower apparent molecular mass, as expected for deletion of seven residues from the C terminus. The 6G6A Rieske protein also migrated at a slightly lower position.

Mutant Rieske Proteins Bearing a Less Flexible Hinge Region or a Deletion of the C Terminus Show Electron Transport Activities Similar to Those of the Wild Type

6G6A, *G171och* (Figure 4), and the control mutant *i1Bam* (data not shown) had wild-type fluorescence characteristics. The *S128T* mutant showed a fluorescence pattern somewhat different from wild type, but nevertheless its LEF approximated that of the wild type, indicating functional b_6f complexes. These data (summarized in Table 2) are consistent with restoration of photosynthetic electron transport and phototrophic growth by nuclear transformation with the mutant *petC* alleles. The functional properties of these mutant Rieske proteins were analyzed further by flash-induced, time-resolved spectroscopic measurements in vivo. As shown in Table 2, values for both phase b of the 515-nm electrochromic band shift and the rereduction kinetics of cytochrome *f* fell largely within the range of values expected for wild-type Chlamydomonas cells (Finazzi et al., 1997). These data indicate that both of the major reactions of the cytochrome b_6f complex, the transfer of an electron through the high-potential chain (Rieske 2Fe–2S→cytochrome *f*→plastocyanin or cytochrome c_6→PSI), and the transfer of a charge across the membrane operated essentially with wild-type kinetics in the *petC* mutants.

DISCUSSION

Substitutions Y87D and W163R Impede Rieske Protein Assembly and Function

Chlamydomonas is the only genetically tractable eukaryote in which photosynthesis and carbon assimilation mutations are conditional rather than lethal. We have used a mutant lacking the Rieske protein (*petC-Δ1*) to develop site-directed mutagenesis of the nuclear-encoded, chloroplast-targeted Rieske protein in Chlamydomonas. One previous mutant, *ac21*, was identified as lacking the Rieske protein and shown to accumulate the remaining subunits of the b_6f complex (Lemaire et al., 1986). We have shown here that *ac21* is indeed a Rieske mutant carrying the substitution W163R. However, the *petC-W163R* mutant protein can accumulate under suitable conditions, integrate into the thylakoid membrane, and transfer electrons, although 10 times more slowly than does the wild-type Rieske protein.

Rieske mutant *794* also carried an aromatic substitution (Y87D) and accumulated only small amounts of the Rieske protein assembled into the b_6f complex. Figure 9A shows the locations of the Chlamydomonas Y87D and W163R substitutions in the structure of the Rieske protein soluble domain. In the spinach protein, Y89 (the counterpart of Chlamydomonas Y87) occupies the gap between the cluster binding fold and the larger subdomain of the soluble fragment. Y89 may form hydrogen bonds with either V114 or F116 (Chlamydomonas V112 or F114) of the cluster binding fold (Figure 9B). The negatively charged aspartic acid substitution at this position is predicted to leave the gap unoccupied, to increase its polarity, and to prevent hydrogen bonds to the cluster fold (Figure 9C). This detachment of the cluster binding fold from the base fold may resemble that recently described in the intermediate Rieske protein state in bc_1 crystals (Iwata et al., 1998). The Chlamydomonas Y87D substitution had a marked effect, presumably because it destabilized the soluble domain fold and allowed accessibility to proteases, resulting in degradation. The W163R substitution of mutant *ac21* similarly replaced an aromatic residue

Table 3. Oligonucleotides Used for Site-Directed Mutagenesis

Name	Restriction Site Created	Corresponding Positions[a]	Sequence[b]
i1Bam	BamHI	—	GCGTATGTGGATcCGGCTTGCACC
6G6A	NotI	s G45-T50 b 63 to 72	GTTCCTCTTTACAGCTCTGcCGcTGcgGcCGcTGcCCAGGCCGCTAAGGACG[c]
S128A	NcoI	s S130 b S163	GTGCCCTTGCCAtGGCgCTCAGTACAACGC
S128C	NcoI		GTGCCCTTGCCAtGGCTgTCAGTACAACGCTG
S128T	NcoI		GTGCCCTTGCCAtGGCaCTCAGTACAACGC
W163A	ScaI	s W164 b none	CTGGCCTGGTCACCTTCagtACtgcGACGGAGACTGACTTCCG
G171och	AflII	s G171-A178 b none	GGAGACTGACTTCCGTACttaagTGGAGCCCTGGTGCGC[d]

[a] Refers to corresponding residues in spinach chloroplast (s) and bovine mitochondrial (b) Rieske proteins based on sequence alignments and the structures of Rieske proteins (Tian et al., 1998). The dash indicates the restriction site created is in an intron.

[b] Sequences are written 5′ to 3′. Lowercase letters indicate modified bases, and underlining shows the locations of newly created restriction sites.

[c] Replaces six Gly residues (45 to 50) with six Ala.

[d] Creates an ochre stop codon resulting in deletion of the C-terminal residues G171 to A177.

with a charged residue. Its phenotype was like that of *petC-Y87D* but less severe in that more of the protein assembled into a b_6f complex. The W163R substitution has a more peripheral location. In spinach, W164 (Chlamydomonas W163) forms hydrogen bonds with the tail region, including a hydrogen bond between its backbone and W177 (Chlamydomonas W176). However, we report here that deletion of residues G171 to A177 had no functional impact in Chlamydomonas.

The Rieske protein is synthesized in the cytosol with an N-terminal signal peptide that directs its import into the stroma of the chloroplast and then is inserted into the thylakoid membrane. The Chlamydomonas chloroplast Rieske protein is easily extracted from the membrane (Breyton et al., 1994) but is most probably anchored in the membrane by an N-terminal α helix with the majority of the protein in the lumen by analogy to the bc_1 complex structure (Kim et al., 1998; Zhang et al., 1998). Stromal proteases have access to the Rieske protein during its translocation and also can cleave a short stromal-exposed segment of the assembled Rieske protein in spinach (Karnauchov et al., 1997). The mutant W163R Rieske protein accumulated less label than did the wild-type protein during short pulse-labeling experiments (Figure 1C). It is improbable that synthesis of the mutant protein was diminished; more probably, however, translocation or stability was affected.

A chloroplast mutant attenuating the expression of the stromal protease ClpP has been constructed (W. Majeran, F.-A. Wollman, and O. Vallon, unpublished results). The double mutants *petC-W163R clpP-AUU* and *petC-Y87D clpP-AUU* reported here had significantly increased quantities of the mutant Rieske proteins compared with the single mutants *petC-W163R* and *petC-Y87D.* Lower labeling and ClpP sensitivity of the mutant proteins are compatible with translocation of the lumenal domain of the Rieske protein across the thylakoid membrane by a ΔpH-dependent mechanism (Madueño et al., 1994) in a prefolded, cofactor binding conformation as suggested by Berks (1996). Less efficient translocation of more open structures of the W163R and Y87D Rieske proteins could allow stromal degradation of a portion of these molecules. Some Y87D and W163R proteins escaped degradation and assembled into b_6f complexes that showed charge translocation (phase b) and cytochrome *f* reduction kinetics ~10- to 20-fold slower than the wild type (Table 2). Cpn60 and heat shock Hsp70 chloroplast-localized chaperones have been reported to interact with the Rieske protein in the stroma (Madueño et al., 1993). Overexpression of Hsp70 (Schroda et al., 1999) may represent a further strategy for increased accumulation of mutant proteins.

Functional Organization of the Thylakoid Membrane in a Stable *PetC* Deletion Mutant

Mutant *788* showed a two-base deletion in *PETC* leading to a frameshift in codon 7 and termination at codon 53 (86 bases before the second intron) that entirely eliminated the Rieske protein. The *PETC* transcript was undetectable in mutant *788.* In eukaryotic cells, mechanisms exist for eliminating mRNAs in which translation terminates prematurely. It has been proposed that any intron located more than 50 to 55 nucleotides downstream of a termination codon can mediate a reduction in mRNA abundance (Nagy and Maquat, 1998). This rule applies to *788* and may explain the absence of *PETC* mRNA from this mutant. In contrast, the

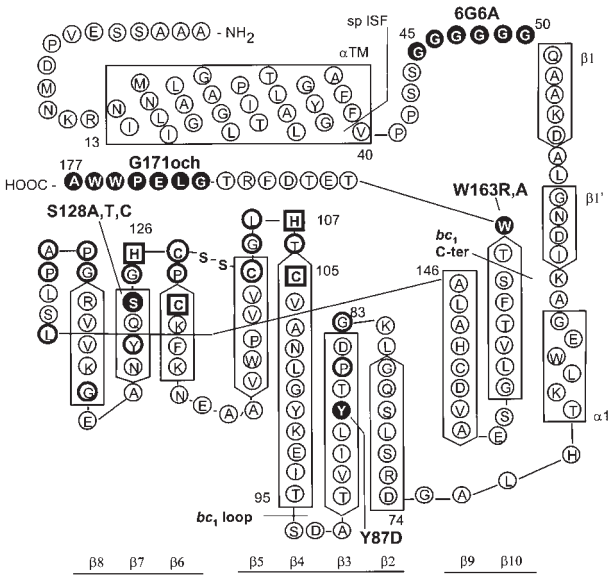

Figure 6. Mutation Sites in the Chlamydomonas Chloroplast Rieske Protein.

Cysteine and histidine ligands for the 2Fe–2S cluster are represented by heavily outlined squares. C110 and C125 form a disulfide bond. The box αTM represents a likely transmembrane α helix. The helix α1 and β sheet strands are indicated, as is the location of the N terminus of the spinach Rieske fragment (sp ISF). Locations corresponding to the C terminus and an extra loop of mitochondrial Rieske proteins are marked bc_1 C-ter and bc_1 loop, respectively. Boldface residues are conserved in the *bc* and *bf* Rieske proteins. Black circles represent sites at which mutations were detected or created. In this work, the model is based on the structures of mitochondrial (Iwata et al., 1996) and spinach chloroplast (Carrell et al., 1997) Rieske fragments and of Rieske subunits of bc_1 complexes (Xia et al., 1997; Kim et al., 1998; Zhang et al., 1998).

PETC transcript overaccumulated in the *petC-W163R* mutant and accumulated to wild-type quantities in the *petC-Y87D* mutant.

Purified b_6f complexes from *788* (*petC-Δ1*) mutant retained a dimer structure, indicating that the Rieske protein does not control the oligomerization of the extractable complex. This is in agreement with experiments on Chlamydomonas mutant *fud2* (Finazzi et al., 1997), which has a duplication of 12 amino acids in the cytochrome b_6 loop between transmembrane α helices C and D of the Q_o binding site. During purification, the Rieske protein detaches from the *FUD2* b_6f complex without leading to monomerization. These observations are of note because loss of the Rieske protein and monomerization often occur simultaneously, which therefore raises the question of their causal relationship (Breyton et al., 1997). The state of oligomerization of nonextractable complexes of the *petC-Δ1* mutant is not known. Extraction of b_6f complexes from thylakoid membranes is less efficient in Rieske protein–deficient mutants (~50% in *petC-Δ1* or *petC-W163R* mutants compared with ~80% in the wild type). This may reflect a modified functional organization of the thylakoid membrane in these mutants.

The "state transition" phenomenon refers to redistribution of antenna proteins between the two photosystems in response to cellular requirements for ATP or reducing equivalents. These transitions do not occur in the absence of the Rieske protein in the *788* (*petC-Δ1*) mutant (R. Delosme, J. Olive, and F.-A. Wollman, data not shown).

The Rieske protein is implicated in a modification of cytochrome b_6. The cytochrome b_6 protein appears upon electrophoresis as two diffuse bands in early exponential-phase cultures (10^6 cells/mL). The lower band is undetectable in the *petC-Δ1* mutant and is restored after transformation with *PetC* alleles. Several distinct immunoreactive bands of cytochrome b_6 have been detected in chloroplast mutants bearing substitutions in one or the other of the histidine ligands for heme b_h (two bands) or b_l (one band) or in nuclear *ccb* mutants defective in holocytochrome b_6 formation (two bands) (Kuras et al., 1997). The absence of the lower band in the *petC-Δ1* mutant suggests that there is a modification of cytochrome b_6 requiring the presence of the Rieske protein.

Structural and Functional Implications of the Site-Directed Mutants

Because of inefficient homologous recombination, directed mutagenesis of the chloroplast Rieske protein depended on a stable, null mutant that could be used as the recipient for modified *petC* alleles. Mutant *788* (*petC-Δ1*), characterized in this work, meets this criterion. As demonstrated by allele-specific PCR and restriction analysis (Figure 7), Chlamydomonas *788* (*petC-Δ1*) mutants transformed with mutant *petC* alleles retained the defective *788 petC* allele and carried, in addition, introduced mutant *petC* alleles. In this study, we have characterized *petC* mutations that restored phototrophy to the *788* (*petC-Δ1*) mutant. Recovery of nonphototrophic transformants should be possible by cotransformation of a *788* (*petC-Δ1*) arg^- mutant with *ARG7* (Debuchy et al., 1989) and *petC* alleles.

Figure 9 shows the locations of the site-directed mutations on the soluble domain structure of the spinach chloroplast Rieske protein. S163 in the bovine mitochondrial Rieske protein forms a hydrogen bond with S-1 of the 2Fe–2S cluster and participates in a hydrogen bonding network that helps stabilize the reduced form of the cluster (Iwata et al., 1996). We constructed the mutations S128A, S128C, and S128T at the corresponding position in the chloroplast Rieske protein (Figure 6). S128T is a conservative substitution designed to test the mutagenesis strategy. The mutations S128A and S128C as well as W163A failed to restore phototrophic growth.

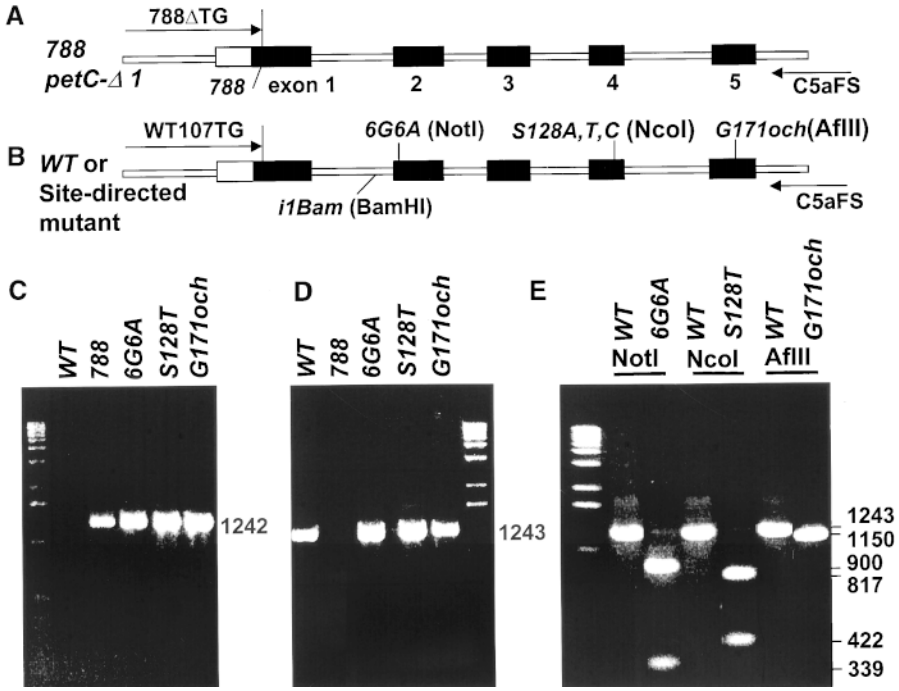

Figure 7. Allele-Specific PCR and Restriction Analysis of Chlamydomonas Transformants.

(A) Map of the *petC* gene of the Rieske deletion mutant *788.* The location of the deletion mutation in mutant *788* is indicated, as are the binding sites for the primers 788ΔTG and C5aFS. The 788ΔTG primer is specific for the *788 petC* allele and, along with primer C5aFS, generates a 1242-bp product by PCR.

(B) Map of the wild-type and site-mutated *petC* genes. Each modified *petC* allele carries a BamHI site (*i1Bam*) in intron 1. The location of each site-directed mutation and its accompanying restriction tag is shown. The WT107TG primer is specific for the wild-type *PETC* sequence of exon 1 and, along with primer C5aFS, generates a 1243-bp product by PCR.

(C) Stained gel showing PCRs with primers 788ΔTG and C5aFS. DNA extracts were from the wild type (*WT*) and Rieske deletion (*788*) mutant, and from mutant *788* transformed with the *6G6A*, *S128T*, and *G171och petC* alleles (fragment sizes in base pairs are shown at right). The left lanes show size standards (1-kb DNA ladder from BRL).

(D) PCRs with primers WT107TG and C5aFS.

(E) Restriction digestion of PCR products obtained with the WT107TG and C5aFS primer set on DNAs from the *788* transformants and the wild type.

Structural analysis of bc_1 complexes (Kim et al., 1998; Zhang et al., 1998) suggests a catalytic domain movement of the Rieske protein that depends on flexibility of the region joining the membrane anchor to the soluble domain. We constructed the mutation 6G6A, which should make the flexible hinge or "neck" region (residues 40 to 50 in Figure 6) less flexible by replacing six glycine residues with six alanine residues. Cyanobacterial chloroplast Rieske proteins contain four to six conserved glycine residues in this region. Our 6G6A mutation had no effect on charge translocation or electron transfer (Table 2). Apparently several various structures of the neck region may provide adequate flexibility. The bc_1 Rieske proteins contain no glycines in this region but do carry three noncontiguous alanines. Recent mutational studies in *Rhodobacter sphaeroides* are consistent with a requirement for flexibility in the neck region (Tian et al., 1998, 1999).

The unique and highly conserved 14- to 17-residue C-terminal extension of cyanobacterial chloroplast Rieske proteins contains several aromatic residues that may play roles in protein stability and perhaps electron transport (Gray and Winkler, 1996; Carrell et al., 1997). To test the role of the b_6f Rieske tail region, we introduced a stop codon at position G171 that deletes seven residues from the C terminus (mutation G171och). Deletions of seven but not 14 residues from the C terminus of an overproduced *Nostoc* Rieske protein still allowed cluster integration in vitro (X. Wu, A. Tsapin, and T. Kallas, unpublished data). Our in vivo seven-residue deletion in the *petC-G171och* mutant showed no detectable functional change from the wild type. Therefore, these unique residues, including the aromatic W176 and W177, play no apparent role in cluster insertion—or in protein stability, electron transport, or subunit interactions in vivo. Interestingly, the site-directed aromatic mutation W163A fell

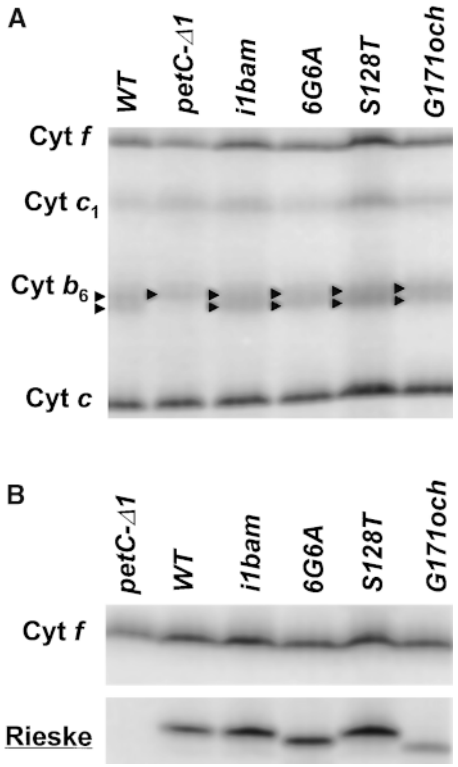

Figure 8. Subunits of the Cytochrome b_6f Complex in Cell Extracts of Site-Directed Mutants.

(A) Heme staining of Chlamydomonas wild-type and mutant cell extracts separated by SDS-PAGE.
(B) Immunodetection of the Rieske protein and cytochrome *f*.
Cyt, cytochrome; *WT*, wild type.

on the same site as mutation W163R in *ac21* mutant. Both *petC-W163R* and *petC-W163A* mutants showed little or no phototrophy. Analysis of the *petC-W163R* and *petC-Y87D* mutants indicates that certain aromatic residues are necessary for Rieske protein stability and assembly, as are specific aromatic residues of the yeast bc_1 protein (Snyder et al., 1999). Furthermore, we find that perhaps as a consequence of structural changes, electron transfer is considerably slowed in these mutants that assemble a portion of the mutant Rieske proteins into only partially functional b_6f complexes. The lack of a more pronounced phenotype in the *petC-6G6A* and *petC-G171och* mutants was surprising. Presumably there are selective reasons for conservation of residues in the flexible hinge and C-terminal extensions of chloroplast Rieske proteins, but these were not apparent from our analyses.

Data presented here establish mutational analysis of the nuclear-encoded and chloroplast-targeted Rieske protein and illustrate the advantages of Chlamydomonas as a model photosynthetic cell.

METHODS

Chlamydomonas reinhardtii Mutants and Genetic and RNA Analyses

Nuclear mutants deficient in the b_6f complex were isolated by using UV mutagenesis of wild-type strain CC125 (*mt*+; Kuras et al., 1997). The *ac21* mutant has been isolated previously (Levine and Smillie, 1962). Strains were grown on Tris-acetate-phosphate medium, pH

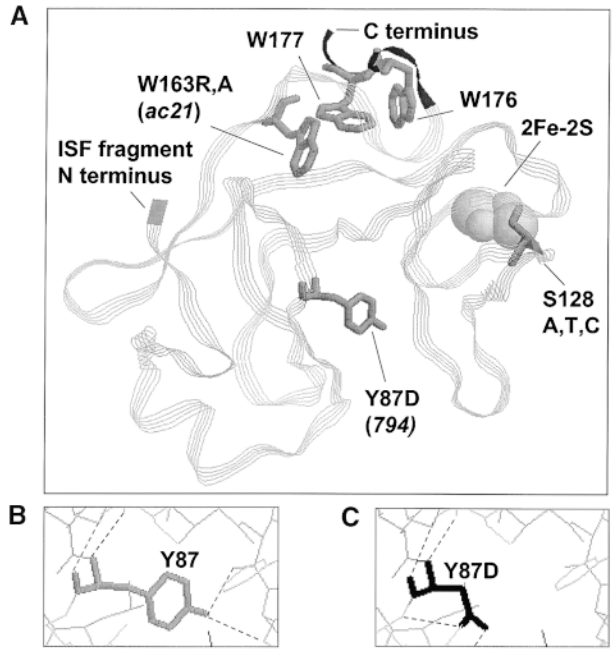

Figure 9. Locations of Mutation Sites in the Soluble Domain of the Chloroplast Rieske Protein.

(A) The structure is that of the soluble fragment (ISF) of the spinach chloroplast Rieske protein (Carrell et al., 1997). The view was generated by the molecular graphics program Rasmol, version 2.6 (R. Sayle, Glaxo, Greenford, UK). Residue numbering is that of the Chlamydomonas protein (de Vitry, 1994). Strands represent the peptide backbone. The 2Fe–2S cluster is shown by van der Waals spheres. Stick structures show Y87 (replaced by aspartic acid residue in the *794* mutant) and W163 (replaced by arginine and alanine residues in *ac21* and *petC-W163A* mutants, respectively). Sticks also represent the mutated S128 and the two tryptophan residues of the C terminus contained within the deletion (shown as a dark ribbon) created by the G171och mutation.
(B) Detail of the Y87 region (equivalent to spinach Y89). Increasing polarity of amino acid residues is represented from light gray to dark gray and possible hydrogen bonds as dashed lines.
(C) Predicted detail of the region shown in **(B)** after substitution with Y87D. The molecular graphics program Swiss-PdbViewer, version 3.0 (N. Guex and C. Peitsh, Geneva Biomedical Resarch Institute, Geneva, Switzerland), was used to visualize amino acid side chain modifications.

7.2, at 25°C under dim light (5 to 6 μE m^{-2} sec^{-1}). Crosses, complementation analyses between nuclear mutants, and reversion and recombination tests were performed according to Kuras et al. (1997). Total RNA analysis was performed according to Drapier et al. (1992).

Plasmid Construction, Site-Directed Mutagenesis, and Nuclear Transformation

Molecular cloning techniques were performed as in Sambrook et al. (1989). *Escherichia coli* DH5α, DH5αMCR (Gibco BRL, Gaithersburg, MD), and ES1301 (Promega, Madison, WI) were grown in Luria–Bertani medium supplemented as needed with 150 μg/mL ampicillin or 15 μg/mL tetracycline. Plasmids were introduced into *E. coli* by electroporation (Gene Pulser apparatus; Bio-Rad) at 12 kV/cm, 25 μF, and 200 Ω settings. Plasmid pACR4.5 was constructed by joining a 4.5-kb BamHI DNA fragment containing the Chlamydomonas *PETC* gene to BamHI-digested, dephosphorylated plasmid pALTER-1 (Promega). Plasmid pACRi1Bam was created by site-directed mutagenesis (altered-sites procedure; Promega) of pACR4.5 with the oligonucleotide i1Bam (Table 3). The resulting ampicillin-resistant and tetracycline-sensitive pACRi1Bam plasmid carries a neutral BamHI restriction site tag in intron 1 of the *PETC* gene; it was used as the template for subsequent mutagenesis with the oligonucleotides described in Table 3. Mutant plasmids, which could be detected by restoration of tetracycline resistance, were subsequently screened for the specific restriction site associated with each directed mutation in *PETC.* Oligonucleotides were purchased in desalted form from Oligo Express (Paris, France). Derivatives of plasmid pACRi1Bam carrying mutations in *PETC* were purified from *E. coli* and introduced into the Rieske deletion *788* mutant (*petC*-Δ1) by helium-driven particle bombardment (Kuras et al., 1997). Phototrophic transformants were selected on minimal medium at light intensities of 50 and 200 μE m^{-2} sec^{-1}. Transformant colonies were visible after 1 to 2 weeks.

Allele-Specific Polymerase Chain Reaction and Restriction Analysis of Transformants

DNA templates were purified from 1.0 mL of late-log phase cultures or from colonies (~10 μL of cell volume). Briefly, cells were pelleted (colonies were suspended in 1.0 mL of water and then pelleted), resuspended in 100 μL of sterile water, and lysed by the addition of 300 μL of freshly prepared lysis buffer (100 mM Tris-HCl, pH 8, 0.4 M NaCl, 40 mM EDTA, 100 μg/mL RNase, 2%SDS) and incubated at 50°C for 15 min. This suspension was extracted with phenol:chloroform:isoamyl alcohol (25:24:1 mixture, v/v/v). DNA from the aqueous phase was precipitated with isopropanol, washed with 70% ethanol, and resuspended in 50 μL of 10 mM Tris-HCl, pH 7.5, and 0.1 mM EDTA buffer. Five-microliter samples were used in 50-μL reactions performed in a Crocodile III thermal cycler (Appligene, Illkirch, France) in a polymerase chain reaction (PCR) buffer containing 20% Qiagen (Chatsworth, CA) Q-solution and Taq polymerase. Amplification products were purified by the Qiaquick method of Qiagen. Allele-specific PCR primers were designed for amplification of the defective *petC*, *788* allele (primer 788ΔTG) 5′-CGCTGCCTCGTCGGAGGC-3′ of mutant *788*, or of the corresponding *PetC* alleles that are wild type in exon 1 (primer WT107TG) 5′-GCTGCCTCGTCGGAGGTG-3′. The same reverse primer (C5aFS) 5′-GAGAGACAATCAACACGCGG-3′ was used with each forward primer. At stringent annealing temperatures of 67 to 68°C, these primers allowed specific amplification and detection of the *788 petC* allele and of wild type or introduced mutant *petC* alleles, both of which are wild type in exon 1.

Fluorescence Induction Measurements and Spectroscopic Analysis of Electron Transfer Kinetics in Vivo

Fluorescence measurements were performed with a home-built fluorometer by using exponential-phase algal cultures maintained in aerobic conditions by vigorous agitation in darkness before experiments. Fluorescence was triggered by continuous actinic light at 590 nm (intensity 125 μE m^{-2} sec^{-1}). F_{max} values were determined in the presence of 10^{-5} M DCMU.

For spectroscopic analysis, algae were harvested in the early exponential phase of growth (2×10^5 to 2×10^6 cells/mL) and resuspended in 50 mM Mops buffer, pH 7 (or 20 mM Hepes buffer, pH 7.2), containing 10% Ficoll, 10 μM DCMU, and 10 mM hydroxylamine to inhibit PSII activity (Joliot et al., 1998). Algae in the cuvette were maintained in anaerobic conditions under a stream of argon. FCCP was added at 1 μM to collapse the permanent transmembrane electrochemical potential. Electron transfer reactions were triggered by an actinic flash from a xenon lamp that was filtered by a dark-red filter (RG695). The intensity of the actinic flash was adjusted to excite less than one PSI per b_6f complex to obtain single turnover of these complexes. The interval between actinic flashes was 8 sec. Kinetics at 515 nm were measured to follow the electrochromic carotenoid band shift, induced by transmembrane charge-transfer reactions. Charge separation in PSI is reflected by a fast rise of the signal that was used to normalize kinetics. The slow phase of the carotenoid band shift was corrected for its decline by a linear approximation of the latter and fitted to an exponential curve to determine the kinetics of b_6f turnover. Cytochrome *f* kinetics were measured at 554 nm and were corrected by subtraction of 3% of the signal at 515 nm for the spectral contribution from the carotenoid band shift at 554 nm. The baseline was assumed to be linear between 545 and 573 nm.

Protein Isolation, Separation, Analysis, and Pulse-Labeling

Biochemical analyses were performed on cells grown to a density of ~2×10^6 cells/mL. For polypeptide analysis, samples were resuspended in 100 mM 1,4-DTT and 100 mM Na_2CO_3 and solubilized in the presence of 2% SDS at 100°C for 50 sec. Polypeptides were separated on 12 to 18% SDS–polyacrylamide gels in the presence of 8 M urea, and heme staining was detected by peroxidase activity of heme binding subunits by using 3,3′,5,5′-tetramethylbenzidine as described by Lemaire et al. (1986). For immunodetection, proteins were electroblotted onto nitrocellulose sheets in a semidry system. Detection by using antisera raised against cytochrome b_6, cytochrome *f*, subunit IV, and ^{125}I-labeled protein A was conducted as given in Breyton et al. (1994) and by using the anti-ClpP antiserum (W. Majeran, F.-A. Wollman, and O. Vallon, manuscript in preparation). Anti-Rieske antiserum prepared against the entire polypeptide was kindly provided by Y. Pierre (Institut de Biologie Physico-Chimique). Thylakoid membrane proteins were purified as in Atteia et al. (1992). Cytochrome complexes were extracted by Hecameg solubilization (at 30 rather than 25 mM) of thylakoid membranes (Pierre et al., 1995).

Whole cells (2×10^6 cells/mL) were pulse-radiolabeled for 7 min as in Lemaire et al. (1986). ^{14}C-Acetate was used in the presence of 100 μg/mL chloramphenicol (an inhibitor of chloroplast protein synthesis)

at 10^{-4} M (5 μCi/mL). Labeling was terminated by dilution of the isotope with 10 volumes of chilled sodium acetate (50 mM).

ACKNOWLEDGMENTS

We thank Sabeeha Merchant for the b_6f mutants; Yves Choquet, Jacqueline Girard-Bascou, Richard Kuras, and David Reardon for help with characterization of Rieske-deficient mutants; Yves Pierre for the anti-Rieske antiserum; Wojciech Majeran and Olivier Vallon for the ClpP-attenuated mutant and anti-ClpP antiserum, crossing *petC-W163R* and *clpP-AUU* mutants, and collaboration in *petC-W163R clpP-AUU* double mutant biochemical analysis; William Cramer for chloroplast Rieske protein coordinates; and Francis-André Wollman for help with fluorescence and ongoing input and support. This work was supported by the Centre National de la Recherche Scientifique (UPR 1261). F.B. was supported by the European Community Fellowship Grant No. ERBFMBICT960707. T.K. acknowledges the U.S. Department of Agriculture (Grant No. 9701960), University of Wisconsin sabbatical, and University of Wisconsin–Oshkosh faculty development grants, and thanks Francis-André Wollman and numerous others at the Institut de Biologie Physico-Chimique for their hospitality. C.d.V. and T.K. acknowledge a Vander Putten international exchange grant.

Received May 14, 1999; accepted August 3, 1999.

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