The small domain of cytochrome *f* from the psychrophile *Chlamydomonas raudensis* UWO 241 modulates the apparent molecular mass and decreases the accumulation of cytochrome *f* in the mesophile *Chlamydomonas reinhardtii*

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Abstract: Cytochrome *f* from the psychrophile *Chlamydomonas raudensis* UWO 241 has a lower thermostability of its c-type heme and an apparent molecular mass that is 7 kDa lower than that of the model mesophilic green alga *Chlamydomonas reinhardtii*. We combined chloroplast transformation, site-directed mutagensis, and the creation of chimeric fusion constructs to assess the contribution of specific domains and (or) amino acids residues to the structure, stability, and accumulation of cytochrome *f*, as well as its function in photosynthetic intersystem electron transport. We demonstrate that differences in the amino acid sequence of the small domain and specific charged amino acids in the large domain of cytochrome *f* alter the physical properties of this protein but do not affect either the thermostability of the c-type heme, the apparent half-life of cytochrome *f* in the presence of the chloroplastic protein synthesis inhibitor chloramphenicol, or the capacity for photosynthetic intersystem electron transport, measured as e^-/P700. However, pulse-labeling with [^{14}C]acetate, combined with immunoblotting, indicated that the negative autoregulation of cytochrome *f* accumulation observed in mesophilic *C. reinhardtii* transformed with chimeric constructs from the psychrophile was likely the result of the defective association of the chimeric forms of cytochrome *f* with the other subunits of the cytochrome b_6f complex native to the *C. reinhardtii* wild type. These results are discussed in terms of the unique fatty acid composition of the thylakoid membranes of *C. raudensis* UWO 241 adapted to cold environments.

Key words: Chlamydomonas raudensis, *Chlamydomonas reinhardtii*, cytochrome *f*, molecular mass, stability, site-directed mutagenesis, chimeric constructs, biosynthesis.

Résumé : Le cytochrome *f* de l'algue psychrophile *Chlamydomonas raudensis* UWO 241 possède une thermostabilité plus faible que l'hème de type c et possède une masse moléculaire apparente de 7 kDa plus faible que celle de l'algue verte mésophile modèle *C. reinhardtii*. Nous avons combiné la transformation de chloroplastes, la mutagenèse dirigée et la création de constructions chimères de fusion afin d'évaluer la contribution de domaines et/ou d'acides aminés spécifiques à la structure, à la stabilité et à l'accumulation de cytochrome *f* ainsi qu'à sa fonction dans le système photosynthétique de transport d'électrons. Nous démontrons que des différences dans la séquence d'acides aminés du petit domaine et la présence d'acides aminés spécifiques chargés dans le grand domaine du cytochrome *f* en modifient les propriétés physicochimiques mais n'affectent ni la stabilité thermique de l'hème de type c, ni la demi-vie apparente du cytochrome *f* en présence d'un inhibiteur de la synthèse des protéines du chloroplaste, le chloramphénicol. La capacité du système photosynthétique de transport électronique mesuré par le e^-/P700 n'est pas affectée non plus. Cependant, un marquage pulsé avec du [^{14}C]acétate combiné avec un immunobuvardage a indiqué que l'autorégulation négative de l'accumulation de cytochrome *f* observée chez l'espèce mésophile *C. reinhardtii* transformée avec des constructions chimères de la psychrophile était probablement le résultat d'une mauvaise association des formes chimères du cytochrome *f* avec les autres sous-unités du complexe cytochrome b_6f originales de *C. reinhardtii* sauvage. Ces résultats sont discutés en termes de composi-

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Abbreviations: cyt, cytochrome; MT, multiple-turnover flash; ST, single-turnover flash,; P700, reduced photosystem I reaction centre; $P700^+$, oxidized photosystem I reaction centre

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tion unique en acides gras des membranes thylakoïdales de *C. raudensis* UWO 241 adaptée aux environnements froids.

Mots-clés : Chalmydomonas raudensis, *Chlamydomonas reinhardtii*, cytochrome *f*, masse moléculaire, stabilité, mutagenèse dirigée, constructions de chimères, biosynthèse.

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Introduction

The structure, composition, and function of the photosynthetic apparatus have been studied extensively in the aquatic, unicellular Antarctic green alga *Chlamydomonas raudensis* UWO 241, with respect to adaptation and acclimation to cold environments (Neale and Priscu 1995; Morgan et al. 1998; Morgan-Kiss et al. 2002*a*, 2002*b*, 2005, 2006; Pocock et al. 2007). Psychrophilic (cold-loving) organisms exhibit optimal growth temperatures below 15 °C but are unable to grow at temperatures above 20 °C (Morita 1975). In contrast, mesophilic organisms, such as the model green alga *Chlamydomonas reinhardtii*, exhibit the capacity to grow at both high and low temperatures, but exhibit optimal growth temperatures in the range of 25 °C to 30 °C (Morita 1975). UWO 241 is a psychrophile that was isolated from the permanently ice-covered Lake Bonney located in the Taylor Dry Valley, Antarctica, the coldest desert on earth (Morgan-Kiss et al. 2006). Consequently, this green alga is adapted to a combination of cold temperatures (0–4 °C), as well as low photosynthetic light flux (5 μmol photons m^{-2} s^{-1}) (Morgan-Kiss et al. 2006). The optimal temperature for growth and photosynthesis for UWO 241 is about 8 °C (Pocock et al. 2007). The thylakoid membranes of this psychrophilic photoautotroph exhibit significantly higher levels of fatty acyl unsaturation in the major thylakoid galactolipids mono- and di-galactosyldiacylglycerol, the primary phospholipid phosphatidyldiacylglycerol (PG), as well as in the sulfolipid sulfoquinivosyldiacylglycerol (SQDG) compared to the model green alga, *Chlamydomonas reinhardtii* (Morgan-Kiss et al. 2002*b*). UWO 241 is unable to grow under red light (Morgan-Kiss et al. 2005), and exhibits unusually low chlorophyll *a* : chlorophyll *b* ratios of 1.8 to 2.1 accompanied by the absence or reduction of all LHCI proteins (Morgan et al. 1998), reduced levels of PS (photosystem) I reaction center proteins (PsaA/B), low PS I – PS II reaction center stoichiometry, but enhanced rates of PS I cyclic electron transport (Morgan-Kiss et al. 2002*a*). Additionally, *C. raudensis* UWO 241 is the first published example of a naturally occurring green alga that is unable to undergo state transitions and phosphorylate LHC II (Morgan-Kiss et al. 2002*a*; Szyszka et al. 2007). Complementation of a *petA* (the gene for cytochrome (cyt) *f*) deletion mutant of *C. reinhardtii* with *petA* from *C. raudensis* UWO 241 showed unequivocally that the difference in the structure of cyt *f* from *C. raudensis* UWO 241 is not responsible for its inability to undergo state transitions and phosphorylate LHC II proteins (Gudynaite-Savitch et al. 2006).

Cyt b_6f, a dimeric membrane protein complex, is a component of the photosynthetic intersystem electron transport chain that is absolutely required for photoautotrophic growth. This complex is composed of 4 major subunits, cyt *f*, cyt b_6, suVI, and Rieske Fe-S protein, and 4 miniproteins of less than 4 kDa (Kurisu et al. 2003; Stroebel et al. 2003). The mature form of cyt *f* in *C. reinhardtii* consists of 2 soluble domains: a highly conserved large domain (residues 1–171 and 229–251) and a small domain (residues 172–228), which is the most variable region among species (Gray 1992). The transmembrane helix (residues 252–271), together with 15 C-terminal amino acid residues residing in the stromal side of the membrane, form the C-terminal domain of cyt *f* (Kuras et al. 1995*a*). The c-heme binding occurs through the formation of 2 thioether bonds between the vinyl side chains of the heme and 2 cysteinyl residues located in the large domain of cyt *f*. The heme binding motif of cyt *f* is part of the longest conserved stretch of amino acids (residues 17–27) (Gray 1992). The N-terminal tyrosine provides the 6th ligand to the heme group. The other conserved amino acid residues, P2, F4, A5, I19, V20, G72, V74, P117, P161, and F237, are involved in shielding the heme from the solvent by forming a hydrophobic pocket (Ponamarev et al. 2000; Chi et al. 2000). Five lysine residues in the large and small domains of cyt *f*, K58, K65, K66, K188, and K189, contribute to a positive electrostatic field that is believed to attract negative charges on plastocyanin, bringing these 2 proteins sufficiently close to allow hydrophobic and van der Waals interactions to form an electron transfer complex (Soriano et al. 1996, 1998; Chi et al. 2000; Gross and Pearson 2003). Mutations within the large domain of cyt *f* inhibit electron transfer (Soriano et al. 1996, 1998; Baymann et al. 1999; Gong et al. 2001), proton translocation (Ponamarev and Cramer 1998), and maturation of cyt *f* (Kuras et al. 1995*a*; Baymann et al. 1999). In contrast, mutations within the variable small domain did not result in any significant effects on electron transfer from that of the wild-type complex (Soriano et al. 1996, 1998). However, the deletion of this domain resulted in slower growth rates and a decreased stability of the cyt b_6f complex (Gong et al. 2001).

The various subunits of the photosynthetic complexes of the thylakoid membrane accumulate through a concerted process that involves 2 types of mechanisms: the efficient post-translational degradation of most unassembled subunits, and an assembly-mediated control of translation of a subset of chloroplast-encoded subunits defined as control by epistasy of synthesis (CES) (Wollman 1998; Wollman et al. 1999; Choquet and Vallon 2000). Cytochrome *f* is one of these CES subunits. The assembly-mediated control of cyt *f* synthesis originates from the autoregulation of the initiation of translation of *petA* mRNA, which involves the 5′ untranslated region of the message (Choquet et al. 1998). In the absence of cyt b_6, subunit IV, or the Rieske Fe-S protein, a nucleus-encoded translational activator, TCA1, which is specific for *petA* mRNA, is likely to be trapped by the C-terminal stromal domain of unassembled cyt *f*, thereby preventing efficient translation of *petA* mRNA (Kuras and Wollman

1994; Wostrikoff et al. 2001; Choquet et al. 2003; de Vitry et al. 2004). The K272 residue in the C-terminal domain has been established as a key residue in the control of cyt *f* synthesis (Choquet et al. 2003). This residue interacts with the N17 residue of the Rieske transmembrane helix via sulfolipid SQDG, which is crucial for the structural integrity and stability of photosynthetic complexes (Riekhof et al. 2003; Stroebel et al. 2003; de Vitry et al. 2004).

The analysis of the major subunits of the cyt b_6/f complex from *C. raudensis* UWO 241 showed that only cyt *f* specifically exhibits an unusually low apparent molecular mass when compared with the subunits of the cyt b_6/f complex from *C. reinhardtii*. This correlates with a lower thermal stability of the c-type heme bound to cyt *f* from *C. raudensis* UWO 241 compared with that of *C. reinhardtii* (Gudynaite-Savitch et al. 2006). Furthermore, *C. raudensis* UWO 241 exhibits a significantly higher level of the fatty acid linolenic acid (18:3), associated with the thylakoid lipids SQDG, PG, and digalactosyldiacylglycerol (Morgan-Kiss et al. 2002*b*). In this report, we combined chloroplast transformation, site-directed mutagenesis, and the design of chimeric fusion protein constructs to assess the contribution of specific domains and (or) amino acids residues to the structure, stability, and accumulation of cyt *f*, as well as its function in photosynthetic intersystem electron transport.

Materials and methods

Cell growth conditions

Chlamydomonas reinhardtii WT.11 wild-type and transformant cells were grown under ambient CO_2 and continuous irradiance (100 μmol photons/m^2 s) in HS medium at 25 °C (Harris 1989). *Chlamydomonas raudensis* UWO 241 was grown in BBM + 0.7 mol/L NaCl at 8 °C, according Morgan-Kiss et al. (2005). The *C. reinhardtii* cyt *f* deletion mutant, Δ*petA*, was grown at 25 °C under ambient CO_2 and continuous light (20 μmol photons / m^2 s) in Tris–acetate–phosphate medium (Harris 1989) containing 100 μg/mL of spectinomycin.

Site-directed mutagenesis and plasmid construction

Four sets of reverse primers were designed to introduce site-directed mutations E10A, P30A, Q50E, and T55K in the mature cyt *f* protein from *C. raudensis* (Table 1). The pair of outside primers, REpetA-F and REpetA-R (Table 1), homologous to *petA* flanking sequences and containing restriction sites *Bgl*II and *Eco*RV, was used with each pair of mutagenesis primers. The plasmid pWF-RApetA, described by Gudynaite-Savitch et al. (2006), containing the construct for expressing the mature cyt *f* from *C. raudensis*, was used as template. Two separate PCR reactions for each site-directed mutation were set by using outside forward with mutagenesis reverse primers for 1 fragment and outside reverse with mutagenesis forward primers for the synthesis of 2 overlapping fragments at the mutation site. All 8 PCR products were gel purified. The pair of fragments for each site-directed mutation was used as a template and as primers in the next 4 PCR reactions, which were prepared as follows. The reaction mixture contained 1 μL of each fragment from each pair, 1× PCR buffer, 0.2 mmol/L dNTPs, and 0.5 μL of HiFi polymerase (Roche Molecular Biochemicals). DNA was denatured at 94 °C for 2 min and the following steps were repeated for 10 cycles: denaturation at 94 °C for 30 s; annealing at 50 °C for 30 s; and synthesis at 68 °C for 45 s. After the last cycle, 1 μL (10 pmol/μL) of each outside primer was added and the standard PCR cycling was continued. The fragments were gel purified, digested with *Bgl*II and *Eco*RV (Fermentas), and ligated into the above-mentioned restriction site in the pWF plasmid, generating the following 4 plasmids: pWF-E10A, pWF-P30A, pWF-Q50E, and pWF-T55K (Fig. 1A). The double mutations were introduced using pWF-P30A for mutagenesis of E10A, and pWF-Q50E for mutagenesis of T55K, thereby generating plasmids pWF-E/P and pWF-Q/T, respectively (Table 1).

Four plasmids, pWF-F1, pWF-F2, pWF-F3, and pWF-F4, containing full-length *petA* fused from different combinations of *petA* from *C. raudensis* and *C. reinhardtii* for complementing cyt *f* in *C. reinhardtii*, were constructed. All plasmids contained the promoter, terminator, signal peptide, and C-terminal stromal domain coding sequence from *C. reinhardtii*. Plasmids pWF-F1 and pWF-F3 contained the *petA* fragment encoding the 1–163 and 1–240 N-terminal domains of mature cyt *f* from *C. raudensis*, respectively, and the remaining C-terminal parts from *C. reinhardtii.* Similarly, the same length of N-terminal parts from *C. reinhardtii* were fused to the C-terminal parts from *C. raudensis* in plasmids pWF-F2 and pWF-F4, respectively (Fig. 1A). Two sets of reverse complement primers, F1/2-F and F1/2-R, and F3/4-F and F3/4-R (Table 1), homologous to the region identical for both algal *petA* genes, were used to construct pWF-F1 and pWF-F2, and pWF-F3 and pWF-F4 plasmids, respectively. To amplify *C. reinhardtii* and *C. raudensis* *petA* fragments, the pWF plasmid, containing wild-type *petA* from *C. reinhardtii* and pWF-RApetA plasmids, were used as the templates, respectively (Gudynaite-Savitch et al. 2006). All plasmids were constructed using the 3-step PCR amplification procedure as described for site-directed mutagenesis using the same set of outside primers, i.e., REpetA-F and REpetA-R.

Chloroplast transformation

The *C. reinhardtii* Δ*petA* mutant, generated from WT.11 by substitution of *petA* by an *aad* cassette (Kuras et al. 1995*b*) was used for transformations. Δ*petA* cells were grown to mid-log phase in TAP medium containing 100 μg/mL of spectinomycin, collected, and prepared for transformation as described by Kuras and Wollman (1994). Gold particles (1.0 μm diameter, Bio-Rad) were prepared for particle bombardment (model PDS-1000/He) according to Boynton and Gillham (1993). For restoring photoautotrophic growth of *C. reinhardtii* Δ*petA* mutant by generating REpetA transformants, the pWF plasmid containing the entire *petA* from *C. reinhardtii* was used. All previously described plasmids containing site-directed mutations in *petA* from *C. raudensis*, i.e., pWF-E10A, pWF-P30A, pWF-Q50E, pWF-T55K, pWF-E/P, and pWF-Q/T, as well as the domain-swapping constructs pWF-F1, pWF-F2, pWF-F3, and pWF-F4, were transformed into the *C. reinhardtii* Δ*petA* mutant, thereby generating the following respective transformants: E10A, P301A, Q50E, T55K, E/P, Q/T, F1, F2, F3, and F4.

Table 1. Primers used for site-directed mutagenesis of cytochrome *f* from *Chlamydomonas raudensis* and domain swapping of cytochrome *f* from *Chlamydomonas reinhardtii* and *C. raudensis*.

Primer[a]	Plasmid	Sequence
REpetA-F	Common for all plasmids	5′-TAAAGATCTTCCATGCATGAAC-3′
REpetA-R		5′-CGCGATATCAACTGCCTCCTTTGGAG-3′
E10A-F	pWF-E10A and pWF-E/P	5′-CAACAAAACTATGctATCCTCGTGAAG-3′
E10A-R		5′-CTTCACGAGGATTagCATAGTTTTGTTG-3′
P30A-F	pWF-P30A	5′-CTTAGCTCAAAAAgCTGTAGAGTTAGAAG-3′
P30A-R		5′-CTTCTAACTCTACAGcTTTTTGAGCTAAG-3′
Q50E-F	pWF-Q50E	5′-GAGGCTATTATCgAAATTCCTTACG-3′
Q50E-R		5′-CGTAAGGAATTTcGATAATAGCCTC-3′
T55K-F	pWF-T55K and pWF-Q/T	5′-TTCCTTACGATAaACAAGTTAAACAAG-3′
T55K-R		5′-CTTGTTTAACTTGTtTATCGTAAGGAA-3′
F1/2-F	pWF-F1 and pWF-F2	5′-GGTCAAGTATATCCAGATGG-3′
F1/2-R		5′-CCATCTGGATATACTTGACC-3′
F3/4-F	pWF-F3 and pWF-F4	5′-AACCCTAACGTTGGTGGTTT-3′
F3/4-R		5′-AAACCACCAACGTTAGGGTT-3′

Note: Nucleotides that differ from those in wild-type sequences are given in lower case letters.

[a]Suffixes -F and -R indicate forward and reverse, respectively.

SDS–PAGE and immunoblotting

Thylakoid membrane polypeptides were isolated from mid-log phase cultures and solubilized as described by Morgan et al. (1998). Samples were loaded on an equal chlorophyll basis (5–10 μg/lane) and electrophoretically separated in a 15% (*w*/*v*) polyacrylamide resolving gel containing 6 mol/L urea, according to Laemmli (1970). Polypeptides separated by SDS–PAGE were transferred electrophoretically to 0.2 μm supported nitrocellulose membranes (Bio-Rad) and immunobloted with the polyclonal antibodies (at a 1:1000 dilution) raised against the 16 C-terminal (cyt *f* C_{ter}) amino acids from spinach cyt *f*, as well as antibodies against entire cyt b_6 and Rieske Fe-S protein from *C. reinhardtii* (Gudynaite-Savitch et al. 2006).

Stability of cyt *f* in the thylakoid membranes of *Chlamydomona*

To inhibit chloroplast protein synthesis, chloramphenicol (100 μg/mL) was added to the exponentially grown cultures of *C. raudensis* UWO 241, *C. reinhardtii* WT.11 and 3e independent RApetA transformants, as described by Gong et al. (2001). To isolate thylakoid membrane polypeptides and immunoblot against cyt *f*, cells were collected every 12 h.

Pulse-labelling experiments

Pulse-labeling experiments were carried out on cells grown to a density of 10^6 cells/mL as described by Kuras and Wollman (1994). In the presence of cycloheximide (8 μg/mL), [^{14}C]acetate was added to cells at a concentration of 5 μCi/mL (1 Ci = 37 GBq) for 5 min. The experiment was stopped by adding 1 volume of ice-cold sodium acetate (50 mmol/L). Proteins from whole cells were solubilized and separated in a 12%–18% (*w*/*v*) polyacrylamide gel containing 8 mol/L urea, as described in Kuras and Wollman (1994).

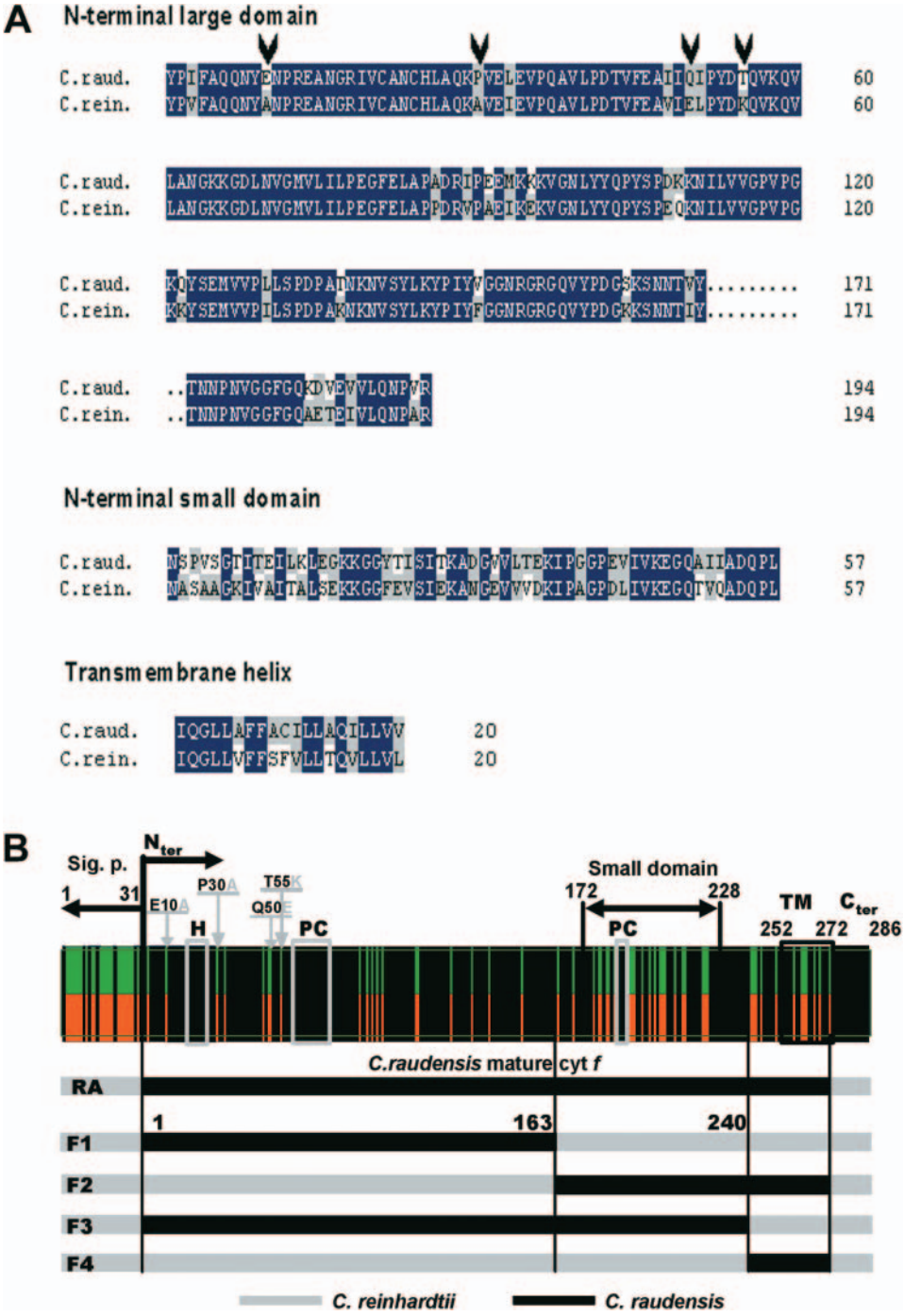
Heme thermostability

Thylakoid membrane proteins were isolated as described previously and suspended into solubilization buffer (Morgan et al. 1998) with a final SDS concentration of 4% (*w*/*v*). After 1 min incubation at 95 °C, aliquots were incubated at 75 °C and sampled at 5 min intervals for a total of 40 min. Polypeptides were separated by SDS–PAGE and transferred onto a nitrocellulose membrane as described above. Heme binding to cyt *f* was visualized by incubation with ECL chemiluminescence detection reagents (Pharmacia Amersham) and developed on X-ray film (Kodak) (Vargas et al. 1993). The intensity of the heme bands after heat treatment was compared with the intensity of bands in samples prior to heating and quantified by densitometry using Scion Image (Scion Corporation).

P700 measurements

The relative redox state of P700 in *Chlamydomonas* cells collected from mid-log phase cultures was determined in vivo under ambient CO_2 conditions using a PAM-101 modulated fluorometer (Walz, Germany) equipped with ED-800T emitter-detector and PAM-102 units following the procedure of Schreiber et al. (1988) as described in detail by Ivanov et al. (1998). Far red light (λ_{max} = 715 nm, 10 W/m, Schott filter RG 715) was provided by the FL-101 light source. Multiple turnover (50 ms) and single turnover (half peak width 14 μs) saturating light flashes were applied with a XMT-103 and XST-103 power control units (Walz, Germany), respectively, via a multibranched fibre optic system connected to the emitter-detector unit and the sample holder. The extent of P700 oxidation was estimated as $\Delta A_{820}/A_{820}$ and recorded using an oscilloscope card (PC-Scope T6420, Version 2.43x; Intelligent Messtechnik GmbH). The complementary areas between the oxidation curve of P700 after single and multiple turnover excitation, and the steady-state levels of $P700^+$ under far red light were used to calculate the ratio of MT/ST which provides an estimate of the intersystem electron donor pool size (e^-/P700) (Asada et al. 1992; Ivanov et al. 2001).

Fig. 1. (A) Amino acid sequence alignment of N-terminal large domain, small domain, and transmembrane helix of cytochrome (cyt) *f* proteins from *Chlamydomonas raudensis* (C.raud.) and *Chlamydomonas reinhardtii* (C.rein.). The protein sequence was predicted from the DNA sequence and aligned using the DNAman program. Black boxes indicate identical amino acids, grey shading indicates similar amino acids, and arrows indicate amino acids substituted by site-directed mutagenesis. (B) Cyt *f* chimeric fusion protein constructs. The schematic alignment of cyt *f* proteins from *C. raudensis* and *C. reinhardtii* is shown on the top, and the black boxes indicate identical amino acid residues. Sig. p., signal peptide; N_{ter}, N terminus of mature protein; H, heme-binding domain; PC, plastocyanin docking site; TM, transmembrane helix; C_{ter}, C terminus. The amino acid substitutions used for site-directed mutagenesis of cyt *f* from *C. raudensis* are indicated with grey arrows above the alignment, where the black letter indicates the amino acid found in wild-type cyt *f* from *C. raudensis* and the grey letter indicates the amino acid found in cyt *f* from *C. reinhardtii*. Schematic sequences of the construct for expression of wild-type mature cyt *f* from *C. raudensis* (RA) and cyt *f* fusion proteins (F1–F4) are shown below the alignment as bars, where grey indicates amino acid sequence from *C. reinhardtii* and black indicates amino acid sequence from *C. raudensis*.



Results

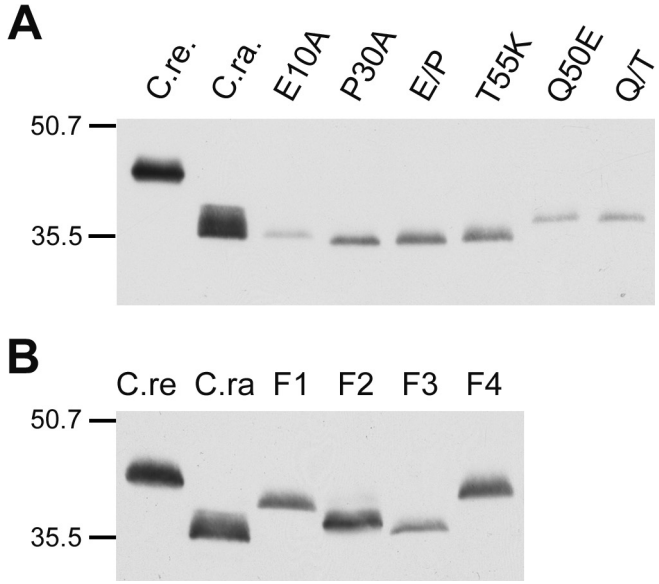
Site-directed mutagenesis of the N-terminal large domain

Previously we suggested that the apparent molecular mass of cyt *f* from *C. raudensis*, which is 7 kDa lower than that from *C. reinhardtii*, is the result of subtle differences in amino acid sequences resulting in different protein structure and folding (Gudynaite-Savitch et al. 2006). The sequence conservation of cyt *f* proteins from *C. raudensis* and *C. reinhardtii* is variable through the whole length of mature protein (Gudynaite-Savitch et al. 2006). The highest conservation, with 87% identity in amino acid sequence, was found in the N-terminal large domain (Fig. 1A). Only 4 amino acids, at positions E10, P30, Q50, and T55 in the large domain of cyt *f* from *C. raudensis*, were significantly different from that of *C. reinhardtii* (Fig. 1A, arrows). To evaluate the potential effects of these amino acids of the large domain of cyt *f* on the apparent molecular mass of this protein, we used site-directed mutagenesis to substitute E10, P30, Q50, and T55 in cyt *f* from *C. raudensis* to the amino acids (A10, A30, E50, and K55) found at the same position in cyt *f* from *C. reinhardtii* and evaluated their effect on the apparent molecular mass of this protein in the *C. reinhardtii* Δ*petA* transformants. To ensure signal specificity for translocation through the thylakoid membrane in *C. reinhardtii* cells, all site-directed mutants harbored the original coding region for the signal peptide from *C. reinhardtii*, as described in Gudynaite-Savitch et al. (2006). Four single (E10A, P30A, Q50E, T55K), and 2 double site-directed mutations (E10A/P30A, and Q50E/T55K) were introduced into the N terminus of the large domain of cyt *f* from *C. raudensis* (Fig. 1B). All mutated cyt *f* proteins from *C. raudensis* complemented the cyt *f* from *C. reinhardtii*, as demonstrated by their ability to grow photoautotrophically. The growth rates of all transformants were lower than those of wild type C. *reinhardtii* and comparable with those of *C. reinhardtii* transformants expressing the wild-type form of cyt *f* from *C. raudensis* (Gudynaite-Savitch et al. 2006; data not shown) However, some of the site-directed mutant proteins showed an apparent molecular mass that was different from the apparent molecular mass of both wild-type cyt *f* proteins (Fig. 2A). The most significant effect the on the apparent molecular mass of cyt *f* from *C. raudensis* was observed with the Q50E substitution, which resulted in a slower migration on SDS–PAGE and thus a higher apparent molecular mass when compared with cyt *f* from *C. raudensis* (Fig. 2A). However, the observed apparent molecular mass of the Q50E cyt *f* was still lower than the apparent molecular mass of the wild-type cyt *f* from *C. reinhardtii*. In contrast, the P30A substitution slightly increased the cyt *f* migration on SDS–PAGE, resulting in an apparent molecular mass that was slightly lower than that of cyt *f* from *C. raudensis* (Fig. 2A). The 2 other single amino acid substitutions, E10A and T55K, did not affect the apparent molecular mass of cyt *f* from *C. raudensis* (Fig. 2A).

Chimeric fusion proteins of cyt *f*

The small domain and the transmembrane helix of cyt *f* from *C. reinhardtii* and *C. raudensis* (Fig. 1A) share only 54% and 65% amino acid sequence identity, respectively, and possess a number of significantly different amino acids

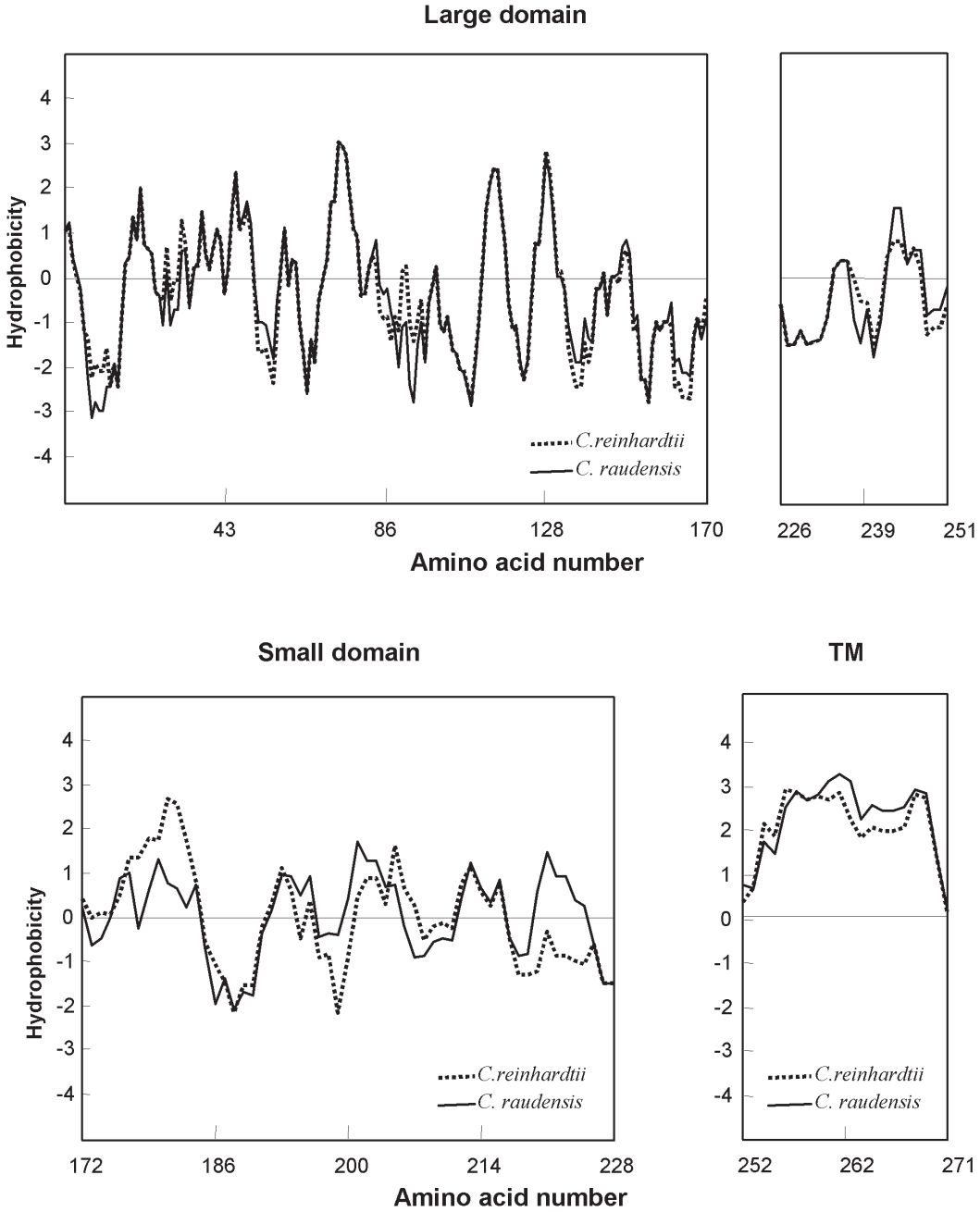
Fig. 2. (A) Immunoblots of cytochrome (cyt) *f* from wild-type *Chlamydomonas reinhardtii* (C.re.), *Chlamydomonas raudensis* (C.ra.), and E10A, P30A, E10A/P30A (E/P), T55K, Q50E, and Q50E/T55K (Q/T) transformants with antibody against the C terminus of cyt *f*. (B) Immunoblots of cyt *f* from wild-type *C. reinhardtii* (C.re.), *C. raudensis* (C.ra.) and F1, F2, F3, and F4 transformants with antibody against the C terminus of cyt *f*. Samples were loaded with equal amounts of chlorophyll (6 μg chlorophyll/lane). The molecular mass markers are indicated on the left side.



when both cyt *f* proteins are compared. Although the large domain exhibited minimal differences in hydrophobicity (Fig. 3), major differences in hydrophobicity patterns of cyt *f* proteins from both *Chlamydomonas* species were associated with the small domain and transmembrane helix (Fig. 3). To evaluate the effects of different domains of cyt *f* from *C. raudensis* on its apparent molecular mass, we made 4 chimeric *petA* constructs, designated F1, F2, F3, and F4 (Fig. 1B). For the fusion points, we used 2 conserved regions, 151–164 and 224–240, which flank the small domain of cyt *f* and are 100% identical in amino acid sequence in both *Chlamydomonas* species (Fig. 1B). All fusion proteins exhibited the same amino acid number and comparable predicted molecular masses as both wild-type cyt *f* proteins (data not shown). Furthermore, the predicted hydrophobicity patterns of the fusion proteins matched the hydrophobicity of the domains from corresponding wild-type species, either *C. raudensis* or *C. reinhardtii* (Fig. 3, data not shown).

Four plasmids (pWF-F1, -F2, -F3, and -F4) containing *petA* encoding the respective fusion proteins, were transformed into the *C. reinhardtii* Δ*petA* mutant. All 4 cyt *f* fusion proteins expressed in *C. reinhardtii* exhibited different apparent molecular masses that were between those of cyt *f* from *C. reinhardtii* and *C. raudensis* (Fig. 2B). As expected, F3 and F4 cyt *f* fusion proteins, containing almost the entire lumenal soluble domains of the cyt *f* from *C. raudensis* and *C. reinhardtii*, respectively, showed an apparent molecular mass that was most similar to that of the corresponding wild-type proteins (Fig. 2B). The F2 fusion protein, containing only the N-terminal large domain from *C. reinhardtii*, fused to the small and C-terminal domains of *C. raudensis*,

Fig. 3. Hydrophobicity patterns of the large and small domains, and the transmembrane helix (TM) of wild-type cytochrome *f* from *Chlamydomonas reinhardtii* (dotted line) and *Chlamydomonas raudensis* (solid line). The relative hydrophobicity patterns were determined using the DNAman program (Kyte–Doolitlle scale, window size 6).



exhibited an apparent molecular mass similar to that of *C. raudensis* cyt *f* (Fig. 2B). However, the F1 fusion protein, containing the small and C-terminal domains of *C. reinhardtii* cyt *f*, exhibited a significantly higher apparent molecular mass than that of *C. raudensis* cyt *f* (Fig. 2B). The similarity of the apparent molecular mass of the fusion proteins F2 and F3 to the apparent molecular mass of the wild-type cyt *f* from *C. raudensis*, as well as the similarity in the apparent molecular masses of the F1 and F4 proteins to wild-type cyt *f* from *C. reinhardtii*, indicated that the small domain of cyt *f* appeared to impart a significant structural effect on cyt *f*. However, exchanging the small domains did not completely convert the apparent molecular mass of *C. raudensis* cyt *f* to that of the *C. reinhardtii* form (Fig. 2B).

The effects of site-directed mutagensis and domain swapping on the accumulation and biosynthesis cyt *f*

We have shown previously that the transformation of *C. reinhardtii* with *petA* from *C. raudensis* results in a decreased relative abundance of the major subunits of the cytochrome b_6f complex, cyt *f*, cyt b_6 and Rieske Fe-S protein (Gudynaite-Savitch et al. 2006). To assess the effects of site-

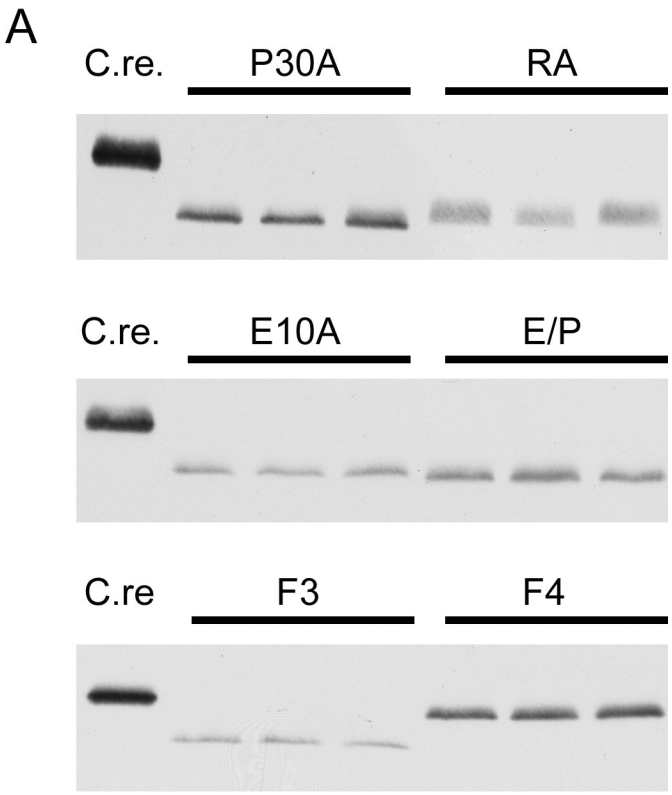


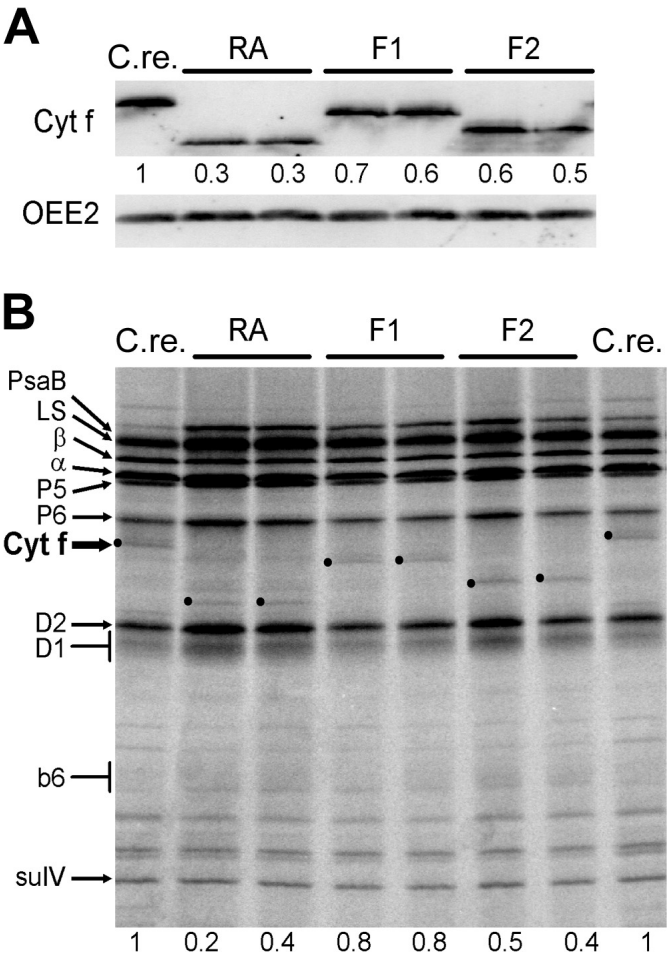
Fig. 4. (A) Abundance of cytochrome (cyt) *f* in *Chlamydomonas reinhardtii* wild-type and transformant cells. Immunoblots blots of thylakoid membrane polypeptides from *C. reinhardtii* wild-type (C.re) and *C. reinhardtii* transformants expressing wild-type cyt *f* from *Chlamydomonas raudensis* (RA), *C. raudensis* cyt *f* with site-directed substitutions (P30A, E10A, and E10A/P30A (E/P)), and domain swapped proteins (F3, F4) with antibodies against the C terminus of cyt *f*. Samples were loaded with equal amounts of chlorophyll (6 μg chlorophyll/lane). (B) Stability of cyt *f* in thylakoid membranes. Immunoblots of thylakoid membrane polypeptides from wild-type *C. reinhardtii* (C.re.), *C. raudensis* (C.ra.) cells, and *C. reinhardtii* RApetA transformants (RA1, RA2, and RA3), with the antibody against the C terminus of cyt *f*. Samples were collected every 12 h after adding 100 μg chloramphenicol/mL to exponentially growing cells and loaded with equal amounts of chlorophyll (6 μg chlorophyll/lane). The film exposure time for immunoblots of the cyt *f* from RApetA transformants was adjusted to get bands with intensities that were comparable with those of wild-type cells.

directed mutagensis and domain swapping on the accumulation of cyt *f*, we compared the abundance of cyt *f* in *C. reinhardtii* wild type and transformant cells expressing either the wild type or site-directed mutants of cyt *f* from *C. raudensis*, as well as domain swapped proteins (Fig. 4A). All transformants tested exhibited a lower abundance of cyt *f* (Fig. 4A) and a concomitant decrease in abundance of cyt b_6 and the Rieske Fe-S protein (data not shown) when compared with that of wild-type cyt *f* in *C. reinhardtii*.

The observed differences in abundance of cyt *f* might be associated with the lower stability of this protein in thylakoid membranes of transformed cells as it was demonstrated for cyt *f* small domain deletion mutants (Gong et al. 2001). To test whether the proteolytic susceptibility of the foreign cyt *f* in thylakoid membranes of *C. reinhardtii* was different from that of native cyt *f* proteins in *C. reinhardtii* and *C. raudensis* wild-type cells, chloroplast protein synthesis was blocked by adding chloramphenicol to exponentially growing cultures of wild-type *C. raudensis* and *C. reinhardtii*, as well as to 3 independent RApetA transformants, RA1, RA2, and RA3 (Fig. 4B). No significant changes could be detected in the stability of cyt *f* inserted into either the thylakoid membranes of the 2 wild-type *Chlamydomonas* species or the transformants. In all instances, a similarly slow decrease in the abundance of cyt *f* was observed, which remained at about 30%–40% of its original level after 72 h (Fig. 4B).

However, the defective association of cyt *f* with the other complex subunits in thylakoid membranes might induce negative autoregulation of cyt *f* biosythesis by the unassembled C-terminal domain (Wostrikoff et al. 2001; Choquet et al. 2003). To address this possibility, we examined the impact of *C. raudensis* wild-type and domain-swapped proteins of cyt *f* on its biosynthesis and assembly into the cyt b_6f complex in 3 *C. reinhardtii* transformants, RApetA, F1, and F2 (Fig. 5). Since the 15 C-terminal amino acids were identical in all constructs of cyt *f* (Fig. 1B), the relative abundance of cyt *f* fusion proteins was evaluated by immunoblotting with antibodies raised against the 15 amino acid C terminus of cyt *f*. As indicated in Fig. 5A, the amount of cyt *f* from *C. raudensis* in *C. reinhardtii* RApetA transformants was about 70% lower than that in wild-type *C. reinhardtii* cells. Although the F1 and F2 transformants accumulated comparable amounts of cyt *f* that were about 35%–45% those of wild type *C. reinhardtii* cells, these transformants accumulated higher levels of cyt *f* protein than the RApetA transformant (Fig. 5A). The differential accumulation of cyt *f* in RApetA, F1, and F2 transformants relative to wild-type *C. reinhardtii* appears to be specific for cyt *f*, since no differential accumulation was observed for OEE2, a subunit of the oxygen evolving complex of PS II (Fig. 5A). To assess the rates of cyt *f* biosynthesis, chlor-

Fig. 5. Abundance of cytochrome (cyt) *f* in *Chlamydomonas reinhardtii* wild-type cells, as well as F1 and F2 transformant cells. (A) Immunoblots of whole-cell polypeptides from wild-type *C. reinhardtii* (C.re.) and 2 independent RApetA (RA), F1, and F2 transformants with antibody against the C terminus of cyt *f* and OEE2. Samples were loaded with equal amounts of chlorophyll (15 μg chlorophyll/lane). The relative abundance of cyt *f* was normalized to that of the OEE2 protein and is indicated under the immunoblot. (B) Pulse labeling of chloroplast-encoded polypeptides with [^{14}C]acetate for 5 min. The gel was loaded with protein extracts from wild-type *C. reinhardtii* (C.re.) and 2 independent RApetA, F1, and F2 transformants. Relative rates of synthesis of cyt *f* were normalized to that of wild-type cyt *f* and are indicated under each corresponding lane.



oplast-encoded polypeptides in the RApetA, F1, and F2 transformants were pulse labeled with [^{14}C]acetate for 5 min. We found no evidence for a loss of correlation between the rates of synthesis and the accumulation of cyt *f* in wild-type *C. reinhardtii* and all transformants tested (Fig. 5B). Indeed, the rate of labeling decreased in the various transformants in the same proportion as their rate of accumulation (Fig. 5), indicating a decrease in rates of biosythesis of cyt *f* in the RApetA, F1, and F2 transformants compared with that of the *C. reinhardtii* wild type.

Table 2. Thermostability of cytochrome (cyt) *f* and the capacity for intersystem electron transport in *Chlamydomonas reinhardtii* wild-type cells (WT.11) and *C. reinhardtii petA* transformants expressing wild-type cyt *f* from *Chlamydomonas raudensis* (RApetA) and cyt *f* fusion proteins (F1, F2, F3, and F4).

Cell type	Heme peroxidase activity (% of control)	e^-/P700
WT.11	49.9±2.8	19.9±1.9
RApetA	17.4±2.7	15.7±1.3
F1	16.2±1.8	13.5±0.9
F2	11.4±1.0	14.1±1.4
F3	11.8±1.4	13.6±1.6
F4	13.9±1.1	13.8±1.5

Note: Thylakoid membrane samples were incubated 10 min at 75 °C, separated using SDS–PAGE, and stained for c-type heme. Heme binding to cyt *f* was calculated from the relative intensity of bands using the ScnImage program. e^-/P700 was calculated as described in Materials and methods. All data are expressed as the means ± SE for n = 4–6 experiments for heme peroxidase activity and n = 12–24 experiments for e^-/P700.

The effects of site-directed mutagensis and domain swapping on the stability and function of cyt *f*

Previously, we showed that the thermostability of heme binding to the cyt *f* from *C. raudensis* UWO 241 is lower than that of *C. reinhardtii* (Gudynaite-Savitch et al. 2006). Since we did not detect significant changes in proteolytic susceptibility between *C. raudensis* and *C. reinhardtii* cyt *f* variants when expressed in the latter cell type, we reasoned that changes in abundance of cyt *f* in the transformants might be due to a decreased stability of the heme. *Chlamydomonas reinhardtii* may have developed a specific heme ligation system for its own apocytochrome *f*, which only poorly recognizes the apocytochrome *f* from *C. raudensis*. This would result in the rapid degradation of apocytochrome *f* in the transformants and a loss of a correlation between the abundance of this protein and its rate of synthesis, as demonstrated previously with cyt *f* mutants defective in heme binding (Kuras et al. 1995*a*). Thus, we assessed the effects of different domains (F1, F2, F3, and F4) of cyt *f* on the thermostability of the heme (Table 2). Heme thermostability of the *C. reinhardtii* transformants expressing each of the 4 chimeric constructs was 3- to 4-fold less than that of WT.11, but comparable with that of cyt *f* from *C. raudensis* UWO 241 expressed in *C. reinhardtii* (RApetA) (Table 2).

Cytochrome *f* is an integral component of the cyt b_6f complex which plays an important role in intersystem electron transfer from plastoquinone to PS I. The in vivo capacity for intersystem photosynthetic electron transport can be assessed by monitoring changes in the redox state of P700 ($\Delta A_{820}/A_{820}$), from which one can calculate e^-/P700 (Asada et al. 1992; Ivanov et al. 2001). As noted in Table 2, e^-/P700 was similar for all 4 fusion proteins, which was about 15% lower than that of RApetA and about 30% lower than that of WT.11. Thus, there appeared to be no correla-

tion between the cyt *f* domain exchanged and either the stability of the heme of cyt *f* measured as heme peroxidase activity or the capacity for intersystem electron transport measured as e^-/P700 (Table 2). It is interesting to note that, irrespective of the domain exchanged, both the heme stability of cyt *f* and e^-/P700 were comparable with that of RA-petA rather than WT.11.

Discussion

From the results of site-directed mutagenesis and domain swapping, we conclude that the observed difference in the apparent molecular mass of cyt *f* proteins from *C. reinhardtii* and *C. raudensis* is the result of small but subtle differences in the amino acid sequence of both polypeptides. Our results suggest that the substitution of only 1 amino acid residue in the cyt *f* from *C. raudensis* to the amino acid residue found at the same position of cyt *f* from *C. reinhardtii* can affect the migration rate of this protein on SDS–PAGE. The most significant effect on the apparent molecular mass was observed with the Q50E substitution (Fig. 2). Possibly, the decrease in the predicted protein charge and pI by the substitution of the neutral amino acid residue to an acidic residue resulted in a different SDS binding ability of this protein, which led to the slower migration on SDS–PAGE (Gray 1992). Proline inserted in the sequence of a polypeptide usually causes bends that disrupt protein secondary structure (Sauer et al. 1992). The P30A substitution in cyt *f* from *C. raudensis* caused a slight decrease in the apparent molecular mass of this protein when compared with the wild-type cyt *f* (Fig. 2A). The F3 and F4 fusion proteins, constructed by exchanging the large domains plus the transmembrane region of cyt *f* between *C. raudensis* and *C. reinhardtii*, respectively, exhibited an apparent molecular mass comparable with that of the corresponding wild-type cyt *f* (Fig. 2B). In contrast, the F1 and F2 fusion proteins, constructed by exchanging the small domains plus the transmembrane region of cyt *f* between *C. raudensis* and *C. reinhardtii*, respectively, exhibited a shift in apparent molecular mass such that the chimeric cyt *f* construct containing the small domain of *C. raudensis* migrated similarly to cyt *f* from wild-type *C. raudensis*, while the chimeric cyt *f* construct containing the small domain of *C. reinhardtii* migrated similarly to cyt *f* from wild-type *C. reinhardtii* (Fig. 2B). Thus, we suggest that the cyt *f* from *C. raudensis* possesses differential protein folding and SDS binding abilities caused by an additive effect of the subtle but significant differences in the amino acid sequence. Thus, the apparent molecular mass of cyt *f*, which is 7 kDa lower than that from the psychrophile *C. raudensis* UWO 241, can be accounted for by the variation in the amino acid sequence within the small domain, as well as differences in a specific charged amino acid, Q50, in the large domain.

Despite the fact that the cyt *f* fusion proteins (F1, F2, F3, and F4) showed significant differences in the apparent molecular mass of cyt *f* compared with both wild-type cyt *f* proteins, the resulting differences in the protein structure did not affect the thermostability of heme bound to the cyt *f* (Table 2). Thus, the observed lower thermostability of heme bound to cyt *f* from *C. raudensis* (Gudynaite-Savitch et al. 2006) must be the result of the folding of the entire protein, rather than a consequence of differential folding within the N-terminal region containing the heme-binding domain. Alternatively, the difference in thermostability might be the result of a difference in the resistance of the reduced and the oxidized forms of cyt *f* to the denaturating conditions and slower unfolding rates of the reduced protein compared with the oxidized form (Sabahi and Wittung-Stafshede 2002). A functional cyt b_6f complex is an absolute requirement for photoautotrophy (Kuras and Wollman 1994). The fact that we observed minimal effects on e^-/P700 (Table 2) is consistent with the fact that all transformants were photoautotrophic and thus exhibited a functional cyt b_6f complex.

Through the comparison of the abundance of the cyt *f* proteins from *C. raudensis* and *C. reinhardtii* expressed in *C. reinhardtii petA* transformants under the control of a native promoter, we demonstrate that altering the small domain of cyt f decreases the rate of biosynthesis and accumulation of this protein in the cyt b_6f complex of *C. reinhardtii*. The 100% identity in the stromal domain of both cyt *f* proteins suggests that the accumulation of this protein is likely regulated by the same mechanism in both *Chlamydomonas* species (Choquet et al. 1998, 2003). Moreover, the correlation between the abundance of cyt *f*, cyt b_6, and Rieske protein (data not shown) reveals the presence of tight control resulting in a concerted accumulation of cyt b_6f subunits in *petA* transformants (Kuras and Wollman 1994). Additionally, the comparable lifetime of wild-type cyt *f* proteins in the thylakoid membranes, regardless of their origin (Fig. 4), suggests that the degradation of the assembled protein is not responsible for the lower accumulation of cyt *f* from *C. raudensis* when expressed in *C. reinhardtii* cells. Since the *C. reinhardtii* wild-type and the *petA* transformants tested exhibit the same correlation between the abundance of cyt *f* and the relative rate of biosynthesis (Fig. 5), we can also exclude a rapid degradation of the *C. raudensis* apoprotein (Kuras et al. 1995*a*) that could be due to ineffective heme ligation to cyt *f* from *C. raudensis* by the heme-ligation machinery of *C. reinhardtii*. We suggest that a negative feed-back on cyt *f* translation might be the cause for the lower abundance of cyt *f* in the transformants (Choquet et al. 1998, 2003). Differential protein folding might result in decreased rates of association of cyt *f* from *C. raudensis* with other *C. reinhardtii* subunits of the cyt b_6f complex. Such unassembled cyt *f* would interact with the TCA1 factor through its free C-terminal domain and downregulate the translation of *petA* mRNA (Wostrikoff et al. 2001; Choquet and Vallon 2000). Despite the apparent differences in the abundance of the cyt b_6f complex, we were unable to detect any significant differences in the capacity for intersystem electron transport measured in vivo as e^-/P700. Thus, the levels of the cyt b_6f complex present in all transformants (F1, F2, F3, and F4) must maintain rates of linear electron transport sufficient to sustain photoautotrophic growth.

We demonstrate that differences in the amino acid sequence of the small domain and a specific charged amino acid, Q50, in the large domain of cyt *f* alter the physical properties of cyt *f* but do not affect either the stability of the c-type heme or the capacity for photosynthetic intersystem electron transport. The negative autoregulation of cyt *f* biosynthesis observed in *C. reinhardtii* transformants is likely the result of the defective association of foreign cyt *f*

with the other cyt b_6/f complex subunits. However, since the cyt b_6/f complex is an integral thylakoid membrane protein complex, lipids also play a critical role in the structure and function of this complex. In fact, the unique thylakoid sulfolipid SQDG has been shown to be particularly important in maintaining the structural integrity of the cyt b_6/f complex (Riekhof et al. 2003; Stroebel et al. 2003; de Vitry et al. 2004). Although the contents of the major thylakoid lipids of *C. raudensis* UWO 241 and *C. reinhardtii* are similar, their fatty acid compositions are unique. The 18:3 content of SQDG as well as that of digalactosyldiacylglycerol and PG are significantly higher in the psychrophile than in the mesophile, *C. reinhardtii*. Thus, the structural differences between cyt *f* from *C. raudensis* UWO 241 and that of *C. reinhardtii* may reflect adjustments in the structure of the cyt b_6/f complex to the unique fatty acid composition of the thylakoid membranes of the psychrophile adapted to a cold, Antarctic environment (Morgan-Kiss et al. 2002*b*).

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