# Conversion of Cytochrome *f* to a Soluble Form *in Vivo* in *Chlamydomonas reinhardtii*†

Richard Kuras, Francis-André Wollman,* and Pierre Joliot

*Service de Photosynthèse, Institut de Biologie Physico-Chimique, 13 rue Pierre et Marie Curie, 75005 Paris, France*

*Received December 28, 1994; Revised Manuscript Received March 13, 1995*⊗

ABSTRACT: We introduced a stop codon in place of the ATT codon encoding Ile283 (numbered from the Met initiation codon) in the petA gene from *Chlamydomonas reinhardtii*. The resulting protein was expected to be truncated on its carboxy-terminus end, lacking the last 35 amino acids. This region of the polypeptide sequence encompasses a hydrophobic stretch assumed to anchor the protein in the thylakoid membrane. Once introduced in whole cells of *C. reinhardtii* by chloroplast transformation, the modified petA gene expressed a truncated apoprotein which was efficiently converted to a truncated holocytochrome *f*. This protein accumulated in the lumen of the thylakoids in a soluble form. Thus the conversion of preapocytochrome *f* to holocyotchrome *f* does not require an interaction with the membrane through its C-terminus anchor. We show that the rest of the cytochrome *b6f* complex failed to accumulate in the transformants, most probably because of a lack of interaction between soluble cytochrome *f* and the other cytochrome *b6f* subunits. However, soluble cytochrome *f* was still able to donate electrons to photosystem I, which is indicative of its ability to maintain interactions with plastocyanin. The control of the rate of synthesis of cytochrome *f* by the neighboring subunit, suIV (Kuras & Wollman (1994) *EMBO J. 13,* 1019−1027), was not observed with the truncated cytochrome *f*. This observation suggests that either the transmembrane anchor of cytochrome *f* contains a target for the regulation of cytochrome *f* translation by suIV or there is a transient form of membrane-bound cytochrome *f* which is highly sensitive to proteolysis at an early post-translational stage.

Cytochrome *b6f* and cytochrome *bc*1 complexes share a number of functional and structural similarities (for reviews see Cramer et al., 1987; Malkin, 1992). In all instances the mature form of the *c*-type cytochrome of the protein complex is anchored in the membranes by a single transmembrane spanning segment located near its C-terminus. The major part of the protein, about 250 amino acids, folds in an aqueous environment, either in the lumen of the thylakoids for cytochrome *b6f* complexes or in the intermembrane space for cytochrome *bc*1 complexes from Gram-negative bacteria and mitochondria. The conversion of the *c*-type cytochrome to a soluble form *in vitro* was first observed upon purification of cytochrome *f* from cruciferous plants (Gray, 1978). It was attributed to a proteolytic cleavage of the last 33 amino acids of cytochrome *f*, which released the hydrophobic stretch located at the carboxy-terminal part of the protein (Martinez et al., 1992). This spontaneous and limited proteolysis of the protein proved extremely useful for structural studies: a three-dimensional structure of soluble cytochrome *f* from turnip has been solved at atomic resolution after crystallization (Martinez et al., 1994).

Conversion of cytochrome c1 to a soluble form has also been successfully achieved *in vitro* by chymotrypsin proteolysis in *Neurospora* (Li et al., 1981) and *in vivo* by site-directed mutagenesis in *Rhodobacter sphaeroides* (Konishi et al., 1991). In the latter case, the resulting strain was not able to grow photosynthetically although the soluble cytochrome *c*1 remained capable of interaction with its natural oxidant cytochrome *c*2.

Here, we examined the biochemical and functional characteristics of a transformant strain from *Chlamydomonas reinhardtii* in which cytochrome *f* was converted to a soluble form by introducing a stop codon upstream of the last 34 amino acids of the polypeptide sequence. We investigated the electron transfer properties that soluble cytochrome *f* has retained *in vivo*. We also studied the biogenesis of the various cytochrome *b6f* complex subunits in the transformants. In a previous study (Kuras & Wollman, 1994) we had shown that the stoichiometric assembly and accumulation of the various subunits of the cytochrome *b6f* complex from *C. reinhardtii* resulted from the interplay of two regulation processes: a post-translational degradation of the unassembled subunits together with a regulation of cytochrome *f* synthesis by the presence of its neighboring subunits. We therefore wondered whether soluble cytochrome *f* would still interact with the other subunits of the cytochrome *b6f* complex, thus allowing their stable accumulation. In addition, creation of a cytochrome *f* protein truncated at its C-terminal end offered the possibility to understand whether the regulation of cytochrome *f* synthesis occurred at an early translational step or whether it required the translation of its carboxy-terminus anchor.

## MATERIALS AND METHODS

*Cell Growth Conditions.* WT, ΔpetD (Kuras & Wollman, 1994), and transformant strains were grown on Tris−acetate−phosphate (TAP) medium, pH 7.2, at 25 °C under 300 lux continuous illumination.

† This work was supported by the CNRS URA 1187 and EEC Contracts BIOCT-930076 to P.J. and F.-A.W., and by HC&M EEC Contract ERB CHRX CT 920045 to F.-A.W. R.K. was a recipient of a doctoral fellowship from the Ministère de la Recherche et de l'Enseignement Supérieur.

* To whom correspondence should be addressed. Phone: 33-1-43 25 26 09; fax: 33-1-40 46 83 31; E-mail address: Wollman@ibpc.fr.

⊗ Abstract published in *Advance ACS Abstracts,* May 1, 1995.

0006-2960/95/0434-7468$09.00/0 

*Mutagenesis and Plasmids.* Oligonucleotide primers for mutagenesis were synthesized using an LKB DNA synthesizer and purified according to the manufacturer's instructions.

Site-directed mutagenesis was performed in *Escherichia coli* according to the method of Kunkel et al. (1987). The mutated products were assessed by sequencing. Mutagenesis were carried out on plasmid piWF (Kuras & Wollman, 1994). This plasmid encompasses the whole petA coding sequence and its flanking regions. In order to convert piWF into pFI283ST, a 33-base oligonucleotide primer was used to convert the CGTATTCAA region of the petA gene, encoding the $R_{182}I_{183}Q_{184}$ peptide, into CG*CTAG*CAA. This led to a neutral mutation for the $R_{182}$ codon but converted the $I_{183}$ codon into a stop codon and generated a new *Nhe*I restriction site. Plasmid pADFI283ST was constructed using plasmid pUC-atpX-AAD (Goldschmidt-Clermont, 1991) digested by *Eco*RV/*Sma*I. The resulting 1.9 kb restriction fragment, carrying the aadA cassette, was introduced at the *Eco*RV site of plasmid pFI283ST, with the same orientation as that of the petA gene. This *Eco*RV site is located 309 bp downstream the end of the petA coding region in the petA-petD intergenic region while the petD coding region is located 2.6 kb downstream of the petA coding region. In order to construct the vector pFI283STΔQ, bearing both the mutated petA gene and a deletion of the petD gene, we replaced the entire petA-petD intergenic sequence located downstream of the aadA cassette in plasmid pADFI283ST by the 3′-untranslated region of the petD gene. To this end, plasmid pADFI283ST was *Xba*I digested, blunt-ended with Klenow enzyme, and then digested by *Pst*I. This digestion produced a large fragment of 7 kb and a 2.5 kb fragment encompassing both the 3′-untranslated region of the aadA cassette and the sequence of the intergenic petA-petD region. Plasmid pQMAD, encompassing a petD gene region in which the petD coding sequence has been replaced by the aadA cassette (Kuras & Wollman, 1994), was digested by *Dra*II, blunt-ended with Klenow enzyme, and then digested with *Pst*I. This produced a 0.9 kb fragment, encompassing the 3′-untranslated regions of both the aadA cassette and the petD gene. Plasmid pFI283STΔQ resulted from the ligation of this 0.9 kb fragment to the 7 kb fragment obtained by the above-described *Pst*I digestion of plasmid pADFI283ST.

*Strains and Transformation.* Cells from *C. reinhardtii* were transformed with a particle gun as described by Boynton et al. (1988). In a first set of experiments we used, as a recipient strain, the nonphotosynthetic FBE (mt+) mutant strain, which bears a deletion of the petA gene (Vallon et al., 1993). This strain was constructed according to Kuras and Wollman (1994) by replacement of the 1.5 kb *Bgl*II−*Eco*RV fragment encompassing the whole petA gene by the aadA cassette. We recovered no phototrophic transformants upon transformation of FBE with plasmid pFI283ST. We then used plasmids pADFI283ST and pFI283STΔQ with the WT (mt+) as a recipient strain in order to select transformants with a truncated cytochrome *f* able to synthesize (F283ST strains) or not (F283STΔIV strains) subunit IV from the cytochrome *b6f* complex. Spectinomycin resistant transformants were selected on TAP medium in the presence of 100 μg/mL spectinomycin. The transformants lacking cytochrome *b6/f* activity were screened on plates, based on their fluorescence induction kinetics after dark adaptation. These clones were then analyzed for their restriction fragment length polymorphism (RFLP) by DNA filter hybridization. The presence of the *Nhe*I restriction site that we had introduced was taken as indicative of the presence of the $I_{283}$/ST mutation. Colonies displaying the expected RFLP pattern were submitted to a further rounds of subcloning on TAP-spec (100 μg/mL) and to RFLP analysis until they became homoplasmic for the expected mutations. The homoplasmic transformants were then transferred and kept on TAP medium for further biochemical and functional analysis.

*Protein Isolation and Separation.* Cell cultures grown in liquid TAP medium at a density of $5 \times 10^6$ cells/mL were harvested by centrifugation at 4000*g* for 10 min at 4 °C. The following procedures were carried out at 4 °C. Pelleted cells were resuspended in 25 mM HEPES-KOH (pH 7.5)/0.3 M sucrose/5 mM $MgCl_2$ and broken by passing the suspension through a French pressure cell under 4000 psi. The homogenate was centrifuged at 4000*g* for 10 min. In order to collect soluble proteins, the 4000*g* supernatant was spun down at 100000*g* for 60 min to remove small membrane fragments. Soluble proteins retained in the supernatant were then precipitated for 60 min in the presence of 10% TCA, harvested by centrigation at 10000*g* for 15 min, and resuspended in 0.1 M DTT/$Na_2CO_3$. The 4000*g* pellet was used to isolate the thylakoid membrane fraction as described by Chua and Bennoun (1975) with the following modification. After purification on a flotation gradient, the thylakoid membrane fraction was collected with the 1.3 M sucrose layer, diluted with 3 volumes of 5 mM HEPES-KOH (pH 7.5)/10 mM EDTA, and pelleted for 10 min at 40000*g*. In order to remove soluble proteins trapped inside the membrane vesicles, the pellet was given two osmotic chocks. To this end, it was resuspended in $H_2O$ for 40 min, pelleted at 340000*g* for 10 min, and incubated in 0.5 M sucrose/5 mM HEPES-KOH (pH 7.5) for 60 min. This procedure was repeated twice. The final pellet was resuspended in 0.1 M $Na_2CO_3$/DTT. Before electrophoresis, proteins from whole cells and thylakoid membranes or soluble proteins were solubilized in the presence of 2% SDS at 100 °C for 50 s. Polypeptides were separated in the Laemmli system (1970) using 12−18% acrylamide gradients in the presence of 8 M urea. Heme staining was performed according to Thomas et al. (1976). Labeled polypeptides were detected by autoradiography of dried gels. Electroblotting of proteins onto nitrocellulose sheets was carried out according to Towbin et al. (1979). Immunodetection was carried out either according to the protocol of Burnette (1981) or using an enhanced chemoluminescence (ECL) method according to the manufacturer's instructions (Amersham International). Antiserum against PC[1] was a generous gift from S. Merchant (UCLA, USA). Other antibodies were the same as in Kuras and Wollman (1994).

*Pulse-Labeling Experiment.* Whole cells ($2 \times 10^6$ cells/mL) were pulse-labeled according to Delepelaire (1983) for 5 min with [$^{14}$C]acetate at a final concentration of 0.1 μM (5 μCi/mL) in the presence of cycloheximide (8 μg/mL). Labeling was stopped by addition of 1 volume of chilled sodium acetate (50 mM).

[1] Abbreviations: Chl, chlorophyll; $cytf_{mb}$, membrane-bound cytochrome *f*; $cytf_s$, soluble cytochrome *f*; DCMU, dichlorophenyldimethylurea; FCCP, carbonyl cyanide [4-(trifluoromethoxy)phenyl]hydrazone; PAGE, polyacrylamide gel electrophoresis; PC, plastocyanin; PS, photosystem; suIV, subunit IV; TMBZ, 3,3′,5,5′-tetramethylbenzidine.

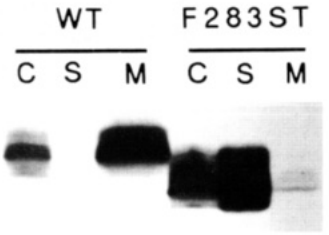


FIGURE 1: Immunoblot showing intracellular distributions of cytochrome f in the WT and the F283ST strains. Whole cell extracts (15 $\mu$g of Chl loaded in lanes C), soluble fraction (load from 30 $\mu$g of Chl cell extract, lanes S), and membrane fraction (45 $\mu$g of Chl loaded in lanes M).

*Spectroscopic Analysis.* Exponentially growing algal cells ($4 \times 10^6$ cells/mL) were concentrated 10 times, resuspended in 20 mM MES-NaOH, pH7/10% Ficoll, and kept in anaerobic conditions in darkness. They were incubated with 20 $\mu$M FCCP in order to collapse the permanent membrane potential and to equilibrate the internal pH with that of the suspension buffer. Absorbance changes due to PSII redox changes were prevented by preilluminating the samples in the presence of $10^{-3}$ M hydroxylamine and $10^{-5}$ M DCMU (Bennoun, 1970).

Spectroscopic measurements were made at room temperature on a spectrophotometer device similar to that described in Joliot and Joliot (1984) and modified as described in Joliot and Joliot (1994). The algae in the measuring cuvette can be rapidly exchanged with those of a larger reservoir maintained in anaerobic conditions.

The redox changes of cytochrome *f* were measured as the difference between the absorption at 553 nm and a base line drawn between 540 and 573 nm, and the redox changes of PC were measured at 573 nm, an isosbestic point for P700 absorption changes. The signal at 573 nm was then corrected for the contribution of cytochrome *f* spectral changes using the following equation: $\Delta I/I$ (PC) = $\Delta I/I$ (573) − (0.21)-($\Delta I/I$ (cytf)). Extinction coefficients at 540, 553, and 573 nm for cytf and at 573 nm for PC were taken from Rich et al. (1987) with values of 20 $mM^{-1}\cdot cm^{-1}$ for cytf at 553 nm and 4 $mM^{-1}\cdot cm^{-1}$ for PC at 573 nm.

The redox changes of P700 were measured as the difference between absorptions at 700 and 729 nm using an extinction coefficient of 70 $mM^{-1}\cdot cm^{-1}$ at 700 nm (Ke, 1972).

## RESULTS

*F283ST Transformants Do Not Grow Phototrophically.* Attempts to select phototrophic transformants using the nonphotosynthetic FBE strain, deleted for the petA gene, as a recipient strain proved unsuccessful. We therefore transformed the WT strain with a construct bearing the selectable marker aadA inserted at the *Eco*RV site downstream of the mutated petA gene (for details see Materials and Methods). As expected, the homoplasmic F283ST transformants, carrying the mutated petA gene, were not capable of phototrophic growth. Their fluorescence induction pattern was indicative of a block in electron transfer from the plastoquinone pool to the PSI reaction centers (results not shown).

*F283ST Transformants Express a Truncated and Soluble Cytochrome.* The presence of cytochrome *f* in WT cells and F283ST transformants was probed on immunoblots with a specific polyclonal antiserum (Figure 1). An immunoreactive

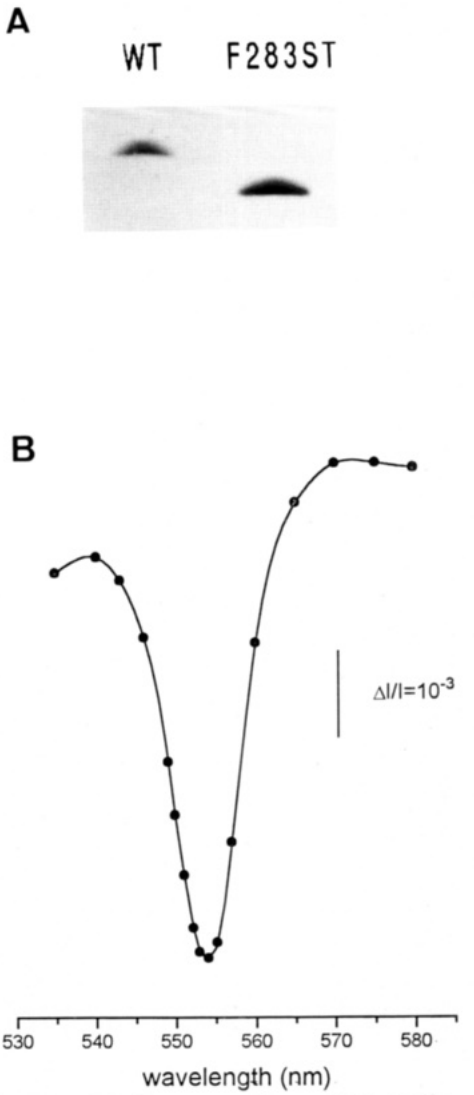


FIGURE 2: Heme binding to cytochrome f. 30−50 kDa region of an electrophoretogram loaded with whole cell protein extracts (15 $\mu$g of Chl) from the WT and the F283ST strains and stained with TMBZ (A). Oxidized minus reduced difference spectrum in the 530−580 nm region, showing spectral properties of photooxidizable $cytf_s$ (B). For experimental details see Figure 3.

protein was detected in both strains (lanes C). However, the comparison of the respective electrophoretic migration positions of cytochrome *f* from the WT and F283ST cell extracts indicated that the latter expressed a truncated version of cytochrome *f*. The apparent molecular mass of cytochrome *f* was 3−4 kDa smaller in F283ST than in the WT as expected from the deletion of the last 35 amino acids of the protein. Total protein extracts from the WT and the F283ST transformants (lanes C) were further separated in a soluble protein fraction (lanes S) and a membrane protein fraction (lanes M) as described in Material and Methods. Cytochrome *f* was totally absent from the soluble protein fraction extracted from WT cells. It was recovered exclusively in the membrane protein fraction (Figure 1, left, lane M). In contrast, cytochrome *f* was recovered in the soluble protein fraction from F283ST transformants (Figure 1, right, lane S). Trace amounts of cytochrome *f* could be detected in the membrane protein fraction (Figure 1, right, lane M). They most likely corresponded to some soluble cytochrome *f* adsorbed on the inner membrane surface and therefore retained in the membrane vesicles created by French press

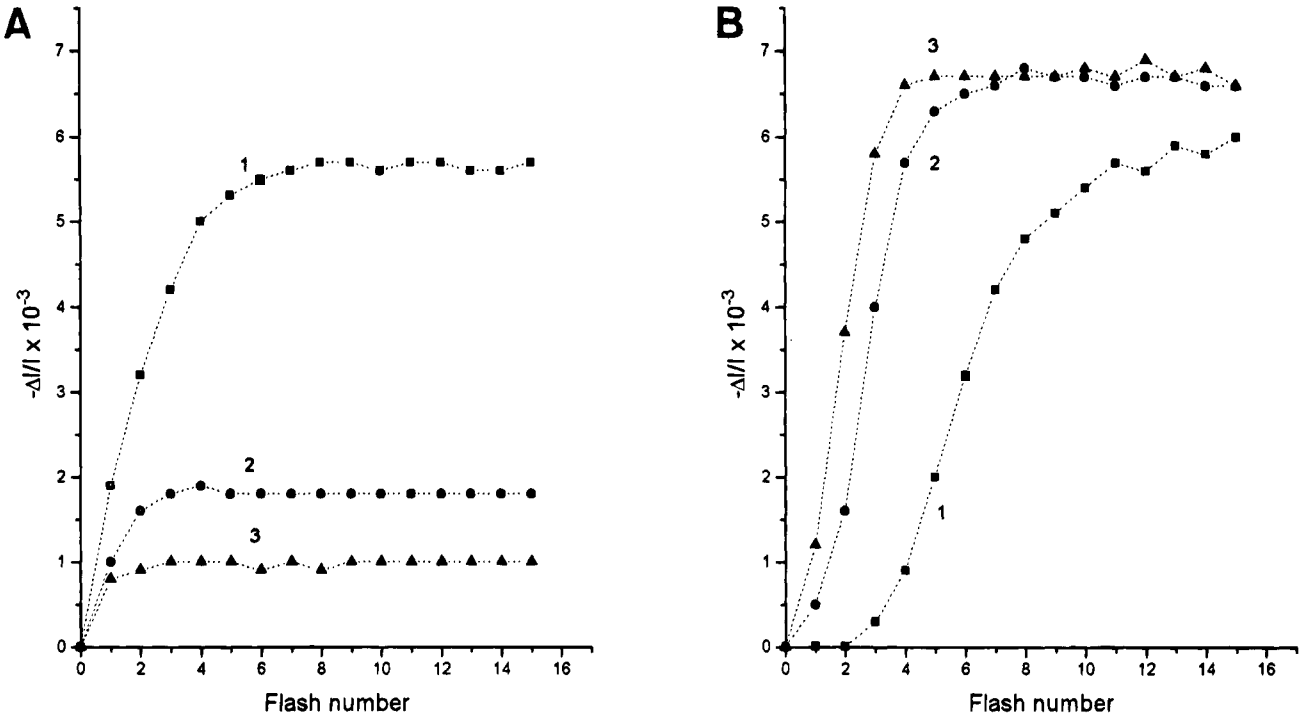


FIGURE 3: Spectral changes measured on algal cells submitted to 3 series of 15 oversaturating flashes. (■, ●, and ▲) Series 1, 2, and 3, respectively. Algae were incubated in anaerobic conditions for more than 1 h in the presence of 5 $\mu$M FCCP. Time interval between flashes: 80 ms. Dark time between each series of flashes: 30 s. One detecting flash was fired 3 ms after each actinic flash. Dark time preceeding the first series of flashes: >5 min. Spectral changes due to cytochrome *f* oxidation: the results are computed from 3 single experiments performed at 545, 554, and 573 nm (A). Spectral changes due to P700 oxidation computed from absorbance differences between 700 and 729 nm (B).

disruption of the intact cells. The two versions of cytochrome *f* will be hereafter referred to as membrane-bound cytochrome *f* ($cytf_{mb}$) for the wild type version and soluble cytochrome *f* ($cytf_s$) for the truncated version in F283ST transformants.

*Soluble Cytochrome f Is Still a Heme-Binding Protein Which Undergoes PSI-Mediated Photooxidation.* Heme binding to cytochrome *f* can be detected on denaturing gels by TMBZ staining. $Cytf_s$ present in total protein extracts of F283ST transformants was also detected by TMBZ (Figure 2A), which indicated that it retained its heme-binding properties. Based on equivalent protein loads, it was even more heavily stained than the original cytochrome *f* from the WT cells. In order to further characterize the heme-binding properties of $cytf_s$, we attempted to establish its oxidized minus reduced spectrum *in vivo*. As will be detailed in the next section, spectroscopic analysis of living cells of the mutant F283ST showed that $cytf_s$ could be fully photooxidized by a series of oversaturating flashes. The spectrum presented in Figure 2B was obtained by plotting the difference bewteen a spectrum recorded 4 s after a series of 20 oversaturating flashes (oxidized $cytf_s$) and a spectrum recorded on dark-adapted material (reduced $cytf_s$). This difference spectrum contained no contribution of P700 redox changes as demonstrated by the absence of absorption changes in the 700−730 nm region (experiment not shown). The spectrum peaked at at 553.7 nm whereas the position of the peak for $cytf_{mb}$ was at 554 nm when measured in the WT strain with the same spectrophotometric technique (experiment not shown). Further analysis with purified $cytf_s$ will be required to establish whether this 0.3 nm shift reflects a genuine change in the spectral characteristics of the truncated cytochrome.

*$Cytf_s$ Re-Reduction Is Impaired in F283ST.* In order to measure the rate of reduction of photooxidized $cytf_s$, algae were illuminated by three series of flashes separated by dark periods of 30 s (Figure 3) . Absorbance changes were detected 3 ms after each actinic flash of a series. The amount of $cytf_s$ oxidized by the second and the third flash series, i.e., 32% and 17%, corresponds to the amount of $cytf_s$ which has been re-reduced during the 30 s dark intervals following the first and the second series of flashes, respectively (Figure 3A). The corresponding half-times for the reduction of $cytf_s$ were then of 50 and 110 s, respectively. Thus the reduction rates for $cytf_s$ were about $10^4$ times slower than for $cytf_{mb}$, which is re-reduced in the millisecond time range (Delosme, 1991).

In a similar experiment, now performed at 573 nm, we observed that PC oxidation was completed within 10−11 flashes of the first series (experiment not shown). Oxidation of P700 required more flashes than that of $cytf_s$ and plastocyanin. As shown on Figure 3B, full oxidation of P700 was achieved only during the course of the second series of flashes. This observation suggested that an additional pool of reductants of unknown chemical nature is able to reduce P700 significantly during the 80 ms dark intervals between flashes in the first series. Figure 4 shows the time course of P700 reduction measured after the last flash of each series of Figure 3. After the first series, P700 reduction followed complex kinetics with two prominent phases showing 4 and 230 ms half-times, respectively. After the second and third series of flashes the rate of P700 reduction was considerably slowed down and became monophasic, with a half-time of about 3 s. We conclude from these experiments that about two flash series are required to exhaust the additional pool of electron donors to PS I, while only ten flashes in the first

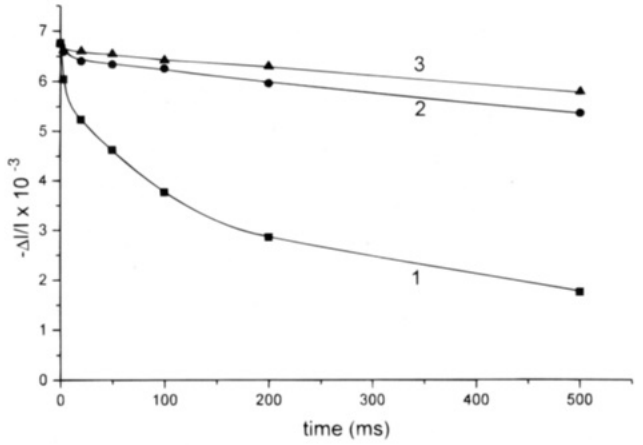


FIGURE 4: Reduction of P700 measured in the dark after the last flash of each series of 15 oversaturating flashes. P700 reduction after the first series of flashes (■), after the second series (●), after the third series (▲).

series were sufficient to fully oxydize $cytf_s$ and PC. Thus, multiple turnovers on the donor side of PSI are severely hampered in F283ST transformants.

*F283ST Transformants Fail To Accumulate the Rest of the Cytb6f Complex but Overexpress $Cytf_s$.* The extent of accumulation of the major subunits of the cytochrome *b6f* complex in F283ST was estimated relative to that in the WT by immunoblotting. The amount of cytochrome *b*6, suIV, and Rieske protein was extensively decreased in F283ST (Figure 5). Comparison of the antibody labeling on decreasing amounts of WT cells with that in F283ST indicated that only 5% of these various cytochrome *b6f* subunits remained in the transformants (experiment not shown). Thus the absence of the rest of the cytochrome *b6f* complex, which accommodates the binding site for the natural reductants of cytochrome *f* (the plastoquinols), provides the molecular basis for the impaired reduction of photooxidized $cytf_s$ in F283ST transformants.

In contrast, $cytf_s$ accumulated extensively in the F283ST transformants (Figure 5). Quantification of its relative concentration was established in two ways. Equivalent protein loads from WT and F283ST strains were immunoblotted and assessed for their content in $cytf_{mb}$ and $cytf_s$, using anti-cytf detection combined with $^{125}$I-protein A binding. The label was quantified by phosphoimaging. This method led to a $cytf_s/cytf_{mb}$ ratio of 2. Alternatively, we calculated the ratios of $cytf_{mb}$/P700 and $cytf_s$/P700 from their respective maximal absorbance changes upon full photooxidation of these species in F283ST and WT strains. The ratio in F283ST transformants can be deduced readily from a comparison of panels A and B of Figure 3. We found a $cytf_s$/P700 value of 2.92. For a similar determination in the WT, algal cells were incubated for 10 min in the presence of 100 $\mu$M benzoquinone and then resuspended in the presence of 10 $\mu$M DCMU, 1 mM hydroxylamine, and 10 mM ascorbate. This pretreatment aimed at a full oxidation of the plastoquinone pool together with a complete reduction of cytochrome *f* in the dark-adapted algae (Joliot & Joliot, 1985). We then illuminated the algae with a series of 20 saturating flashes in order to fully oxidize the primary and secondary PSI donors while PSII was blocked in a reduced state. Absorption changes between the dark-adapted and illuminated samples in the 540−573 and 700−730 nm regions allowed us to measure a $cytf_{mb}$/P700 ratio of 0.88.

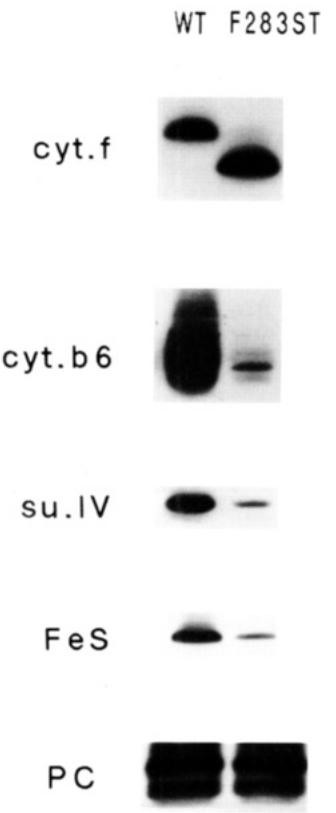


FIGURE 5: Immunoblots of whole cell protein extracts (15 $\mu$g of Chl) from the WT and the F283ST transformant, reacted with antisera against cytochrome *f*, the N-terminus part of cytochrome *b*6, the N-terminus part of suIV, the Rieske iron−sulfur protein (FeS), and plastocyanin (PC). Note that merging of immunoreactions on the diffuse cytb6 band is responsible for apparent overload of the protein in the WT.

Assuming a similar content in P700 in the two strains, we conclude to a $cytf_s/cytf_{mb}$ ratio of 3.3 by the spectroscopic technique. Thus, both the biochemical and spectral determinations showed that the intracellular concentration of $cytf_s$ in F283ST transformants was significantly higher than that of $cytf_{mb}$ in the WT strain. Although we cannot presently rationalize the basis for the difference between the two measurements, their comparison suggests that all $cytf_s$ present in F283ST transformants undergoes photooxidation.

*$Cytf_s$ Does Not Alter the Electron Donation Properties of PC to PSI.* PC oxidation was monitored at 573 nm (see Materials and Methods) in experimental conditions similar to those in Figure 3. We found a total amount of photooxidizable PC per P700 of 2.7 (experiment not shown). This value is close to the PC/P700 stoichiometry of about 3 previously reported in higher plants or algae (Haehnel et al., 1989; Delosme, 1991). It is consistent with the similar amounts of PC that were immunodetected in the WT and F283ST transformants (Figure 5). The respective proportions of PC and $cytf_s$ that were reduced in F283ST during the dark period between the three series of flashes allowed us to estimate the equilibrium constant between the two species (Table 1). We found values of about 2, which indicated that the midpoint potential of $cytf_s$ was 18 mV more negative than that of PC. These values are similar to those obtained for $cytf_{mb}$ vs PC in the WT strain (experiment not shown).

The absence of detectable $P700^+$, 3 ms after each of the two first flashes in series 1 on Figure 3B, indicated that an efficient reduction of $P700^+$ was completed within this time

Table 1: Equilibrium Constant ($K$) Deduced from Absorbance Changes of PSI Electron Donors in F283ST Transformants

| no. | $\Delta I/I$ (cytf)[a] $\times 10^3$ | $\Delta I/I$ (573 nm) $\times 10^3$ | $\Delta I/I$ (PC)[b] $\times 10^3$ | cytf/ cytf$^+$ | PC/ PC$^+$ | $K$ |
|---|---|---|---|---|---|---|
| 1 | 5.68 | 2.26 | 1.04 | | | |
| 2 | 1.79 | 0.89 | 0.5 | 0.45 | 0.94 | 2.09 |
| 3 | 0.97 | 0.52 | 0.31 | 0.20 | 0.43 | 2.15 |

[a] Absorbance changes, corresponding to the maximal oxidation of cytf or PC upon each series of flashes (nos. 1−3), were measured under the same conditions as in Figure 3. [b] After correcting the 573 nm absorbance change for the contribution of cytf.

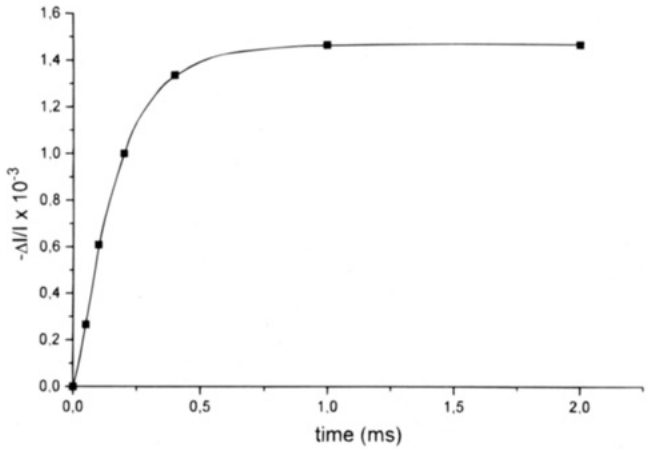


FIGURE 6: Kinetics of cytochrome *f* oxidation measured after a single saturating flash. Same experimental conditions as in Figure 3.

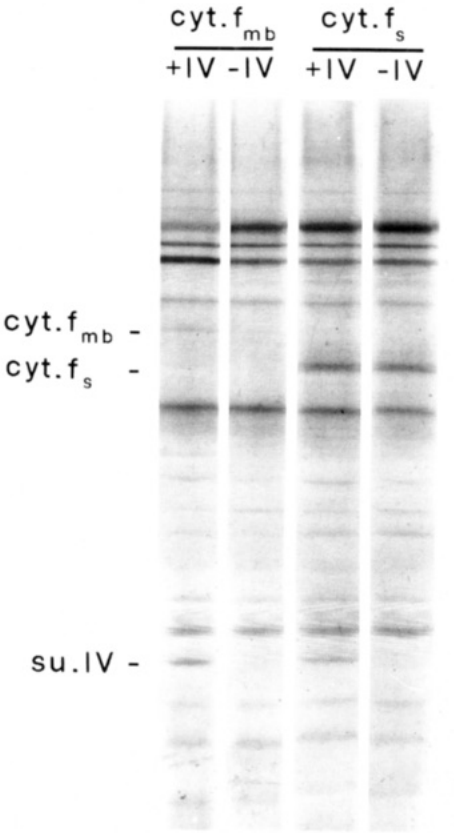


FIGURE 7: Autoradiogram of a urea−SDS−PAGE gel showing chloroplast-encoded proteins pulse-labeled for 5 min in the presence of [$^{14}$C]acetate (5 $\mu$Ci/mL). 15 $\mu$g Chl loads of WT (cytf$_{mb}$, +IV lane), ΔpetD strain (cytf$_{mb}$, −IV lane), F283ST transformant (cytf$_s$, +IV lane), and F283STΔIV transformant (cytf$_s$, −IV lane). Polypeptides are identified by their electrophoretic migration positions, identical to the corresponding immunodetected bands.

range. Delosme has previously shown that PC molecules are associated with about 90% of PSI centers in the WT strain of *C. reinhardtii* and of another green alga *Chlorella sorokiniana* (Delosme, 1991). The half-time of electron transfer between PC and P700$^+$ within these complexes is 4 $\mu$s. In order to get further insights on the time range of P700$^+$ reduction in F283ST transformants, we compared the number of PSI charge separations generated by a short saturating laser flash of 100 ns duration with that obtained with an oversaturating xenon flash which has a tail in the 20 $\mu$s time range. In the latter case, a fraction of PSI centers should undergo re-reduction of P700$^+$ by PC during the time of the flash, thus generating "double hits" where two PSI charge separations occur consecutively within the same reaction center (Delosme et al., 1978). PSI charge separations were measured at 515 nm where the associated change in transmembrane potential induces an electrochromic shift (Junge & Witt, 1968). We found that the number of charge separations induced by the xenon flash was 1.5 times higher than the signal generated by the laser flash in both the WT and F283ST transformants (data not shown). Thus double hits occurred with the same probability in the two strains when using the xenon flash. This observation shows that the presence of both a soluble *c*-type cytochrome and PC in the lumen of the thylakoids in F283ST transformants did not interfere with the rapid reduction of P700$^+$ by PC.

*The Rate of Cytf$_s$ Oxidation by PSI Remains Similar to That of Cytf$_{mb}$.* The time course of cytf$_s$ oxidation was measured after giving a saturating flash to F283ST cells which were dark-adapted in the presence of FCCP (Figure 6). The half-time of the oxidation kinetics was 120 $\mu$s, a value very close to what has been reported previously for cytf$_{mb}$ under the same conditions (Delosme, 1991). In addition, preliminary experiments showed that the rate of electron tranfer between cytf$_s$ and P700 was significantly slower in the absence than in the presence of FCCP, an observation similar to what Delosme (1991) has reported for cytf$_{mb}$.

*The Control of Cytochrome f Synthesis by SuIV Requires Translation of Its Carboxy-Terminus End.* In a last series of experiments, we addressed the question of the possible control of cytf$_s$ synthesis and accumulation by the presence of the transmembrane subunits of the cytochrome *b6f* complex (Kuras & Wollman, 1994). To this end, we fused the construct encoding cytf$_s$ with a construct bearing a deletion of the petD gene encoding suIV. Subsequent transformation of WT cells yields strains expressing the truncated cytf in the total absence of suIV synthesis. Detection of cytf$_s$ by TMBZ staining or immunolabeling showed that the accumulation of cytf$_s$ in these transformants was similar to that in F283ST (experiments not shown).

We then compared the rates of synthesis of cytf$_{mb}$ with those of cytf$_s$ in the presence and absence of suIV synthesis. Whole cells of the WT and the various transformants (ΔpetD, F283ST, and F283STΔIV) were pulse-labeled for 5 min with [$^{14}$C]acetate in the presence of cycloheximide which abolishes cytoplasmic translation. The autoradiogram of the labeled polypeptides separated by SDS−PAGE in the presence of 8 M urea is shown on Figure 7. The deletion of the petD gene (−IV lanes) results in the absence of a labeled band, suIV, with an approximate molecular mass of 17 kDa, otherwise

visible in the WT and F283ST (+IV lanes). Consistent with our immunoblotting detection and TMBZ staining, the two strains expressing a truncated cytochrome *f* displayed a labeled band migrating 3−4 kDa ahead of holocytochrome *f* ($cytf_s$ lanes in Figure 7). Interestingly, this $cytf_s$ band was more heavily labeled in F283ST than the $cytf_{mb}$ band in the WT. Thus the rate of synthesis of a truncated cytochrome *f* lacking its carboxy-terminal anchor in the membrane was higher over 5 min than that of the membrane bound cytf. Moreover, the drastic drop in $cytf_{mb}$ synthesis when suIV is absent (compare $cytf_{mb}$ lanes, +IV vs −IV, in Figure 7) that we have characterized previously (Kuras & Wollman, 1994) was no longer visible for $cytf_s$ in the F283STΔIV transformant: the rate of $cytf_s$ was identical whether suIV was present or absent ($cytf_s$ lanes).

## DISCUSSION

*Biogenesis of Cytochrome f.* The biogenesis of cytochrome *f* encompasses several co- or post- translational steps by which preapocytochrome *f* is converted in holocytochrome *f*. Most of the protein is translocated in the thylakoid lumen, but cytochrome *f* remains associated with the membrane by a single transmembrane spanning segment situated next to its C-terminal end (Gray, 1992). In this study, we converted cytochrome *f* to a soluble form by introducing a stop codon in the petA gene before the sequence encoding the C-terminal membrane anchor. The transformed strain F283ST expressed a truncated protein, $cytf_s$, which still bound a *c*-heme and accumulated as a water-soluble protein in the lumen of the thylakoids. These observations show that membrane binding of cytochrome *f* by its C-terminal end is not required for the protein to be processed, associated with heme, and translocated through the membrane. We conclude that the folding of preapocytochrome *f* as a substrate for the signal peptidase and for the heme lyase does not involve a contribution from the distal C-terminal domain.

We observed however that the rate of synthesis of $cytf_s$ in 5 min pulses was significantly higher than that of $cytf_{mb}$. Changes in *pet*A gene expression in the transformants, at a pretranslational level, are unlikely to be responsible for this observation since there is no direct correlation between transcript levels and translational rates in the chloroplast (Drapier et al., 1992; Wollman & Girard-Bascou, 1994). In addition, we observed that the synthesis of $cytf_s$ was independent of the presence or absence of suIV in contrast with that of $cytf_{mb}$ which is extensively depressed in the absence of suIV (Kuras & Wollman, 1994). Thus the control of $cytf_{mb}$ synthesis by suIV requires the presence of the C-terminal transmembrane anchor. This polypeptide segment could bear a site which modulates the rate at which $cytf_{mb}$ translation is completed or reinitiated. However, the simplest interpretation of these observations is that the C-terminal anchor favors the transient accessibility of *de novo* made cytochrome *f* to a membrane-bound protease. The rapid translocation of $cytf_s$ in the lumen would prevent its early degradation whereas retention of $cytf_{mb}$ next to the membrane by its C-terminal anchor would give access to the protease unless it is rapidly shielded by its assembly with suIV/cyt*b*6 subcomplexes.

The deregulated synthesis of $cytf_s$ was accompanied by a 2−3-fold increase in its steady-state concentration as compared to that of $cytf_{mb}$ in the WT. At variance with the case of $cytf_{mb}$ (Kuras & Wollman, 1994), the same steady-state concentration of $cytf_s$ was reached independent of the synthesis of the other subunits of the cytochrome *b6f* complex, as examplified here by the case of suIV. We conclude that no significant interaction developed between $cytf_s$ and the rest of the cytochrome *b6f* complex since both the rate of synthesis and the accumulation levels of $cytf_s$ were independant of the presence of the other subunits. Consistent with this view, F283ST transformants failed to accumulate the other subunits of the complex as did a strain bearing a deletion of the petA gene, in which stabilization of the rest of cytochrome *b6f* subunits by their assembly with $cytf_{mb}$ was prevented (Kuras & Wollman, 1994). Thus, the C-terminal membrane anchor of cytochrome *f* is a key requirement for the stable assembly of cytochrome *b6f* complex. This observation supports the view that the assembly of transmembrane oligomeric proteins is dependent upon helix/helix interactions (Popot & Engelman, 1990). However, we cannot exclude presently that the transmembrane anchor would merely stabilize an assembly-competent orientation of the lumenal domain of cytochrome *f*.

*Electron Transfer Properties of $Cytf_s$ in Vivo.* Owing to these biogenesis properties, F283ST transformants were non phototrophic since $cytf_s$ could no longer interact, through the Qz site of an assembled cytochrome *b6f* complex, with its natural reductant, the plastoquinol. As a result, the reduction of photooxidized $cytf_s$ was impaired and displayed a half-time slower by 4 orders of magnitude than that of cytochrome *f* in the WT strain. We observed that $P700^+$ could still be reduced with millisecond kinetics when both PC and cytochrome *f* were oxidized. This could originate from a back-reaction occurring once the pool of PSI acceptors is fully reduced. Alternatively, there could be an additional pool of endogenous reductants of presently unknown origin. A possible candidate is the pool of plastoquinol which could have a slow access to PSI in the absence of cytochrome *b6f* complexes. This is supported by the pattern of fluorescence changes at room temperature with *C. reinhardtii* mutants lacking PSII centers. They display a small but significant decrease in their fluorescence yield, in the millisecond time range, upon continuous illumination. This process most likely originates from a PSI-mediated oxidation of the plastoquinol pool. Interestingly, a similar quenching process is still observed with double mutants lacking both the PSII centers and cytochrome *b6f* complexes, although the kinetics are then about 10 times slower (Wollman, unpublished observations).

The failure to assemble cytochrome *b6f* complexes in the transformants led to the degradation of the Rieske protein. This prevented us from understanding whether the Rieske protein *per se* would be able to transfer reducing equivalents to $cytf_s$. Reconstitution experiments, using $cytf_s$ purified from the transformants and the Rieske protein purified from the WT strain, will be required to address this point.

The impaired reduction of oxidized $cytf_s$ is in great contrast with the seemingly unaltered electron transfer properties from $cytf_s$ to PSI. The presence of two soluble electron carriers, $cytf_s$ and PC, in the lumen of the thylakoid, did not interfere with the interaction between PC and PSI centers. PC remained the primary donor to PSI with microsecond kinetics as in the WT. The overall spectral properties and redox potentials of the two species remained unaltered in F283ST transformants.

The rates of oxidation of $cytf_s$ and of $cytf_{mb}$ by PC were similar although the concentration of the former was 3 times higher. Therefore, we conclude that this process is not limited by the diffusion of PC between P700 and $cytf_s$. Delosme (1991) reported that, in anaerobic conditions, the rate of $cytf_{mb}$ oxidation by PSI was much faster in the presence than in the absence of uncouplers which are known to collapse the permanent membrane potential. The two situations resembled those corresponding to state II and state I, respectively (Bulté et al., 1990). Since the proportion of cytochrome *b6f* complexes increases in the PSI region in state II (Vallon et al., 1991), it was suggested that the fast kinetics of cytochrome *f* oxidation could result from the formation of supercomplexes between cytochrome *b6f* and PSI (Delosme, 1991). Thus a first interpretation of the similar oxidation kinetics of $cytf_s$ and $cytf_{mb}$ in the presence of FCCP is that both $cytf_s$ and $cytf_{mb}$ can associate in a ternary complex with PC and PSI.

A second interpretation is that a collisional process induces the formation of a transient complex between $cytf_s$ (resp. $cytf_{mb}$) and PC. Then, the rate-limiting step should be either the electron transfer reaction within the $cytf_s$/PC complex (resp.$cytf_{mb}$/PC complex) or the release of PC from the PC–P700 complex.

The present study shows some similarities with a previous report on the behavior of a truncated and soluble cytochrome *c*1 in the inner membrane system of *R. sphaeroides* (Konishi et al., 1991). In the two cases, the soluble cytochrome kept its electron donation properties although its re-reduction is impaired. The bacterial system involves as an intermediary donor a soluble cytochrome, cytochrome *c*2, instead of a copper-containing electron carrier, PC. In addition, cytochrome *f* and cytochrome *c*1 most likely differ by the nature of the sixth ligand of the *c*-heme (Martinez et al., 1994). In spite of these differences, the properties of the electron transport chain from cytochrome *b6f* to PSI share many similarities with those of the donor side to the reaction center from purple bacteria: in the two cases, once the charge separation has occurred, the photosynthetic membrane is not a requirement for electron transfer reactions between the secondary donors which can develop in an aqueous environment. In the present case, the strikingly similar rates of electron transfer reactions whether the donor side involves $cytf_s$ or $cytf_{mb}$ suggests that the thylakoid membrane plays no topological role in the organization of the donor side of PSI. The fact that uncouplers like FCCP modify rate constants between cytf and PC could be considered as the mere result of pH changes in the lumen compartment. In contrast, anchoring the secondary donor to the membrane is a requirement for its participation to the cytochrome *b*-mediated oxidation of the membrane reservoir of quinols.

## ACKNOWLEDGMENT

We thank S. Buschlen and M. Grevin for technical assistance, and R. Delosme for critical reading of the manuscript.

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BI942967J