Functional Characterization of *Chlamydomonas* Mutants Defective in Cytochrome *f* Maturation*

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We have altered the N terminus of cytochrome *f* by site-directed mutagenesis of the chloroplast *petA* gene in *Chlamydomonas reinhardtii*. We have replaced the tyrosine residue, Tyr^32*, located immediately downstream of the processing site Ala^29*-Gln^30*-Ala^31* by a proline. Tyr^32* is the N terminus of the mature protein and serves as the sixth axial ligand to the heme iron. This mutant, F32P, accumulated different forms of holocytochrome *f* and assembled them into the cytochrome *b*/*f* complex. The strain was able to grow phototrophically.

Our results therefore contradict a previous report (Zhou, J., Fernandez-Velasco, J. G., and Malkin, R. (1996) *J. Biol. Chem.* 271, 1–8) that a mutation, considered to be identical to the mutation described here, prevented cytochrome *b*/*f* assembly. A comparative functional characterization of F32P with F29L-31L, a site-directed processing mutant in which we had replaced the processing site by a Leu^29*-Gln^30*-Leu^31* sequence (2), revealed that both mutants accumulate high spin cytochrome *f*, with an unusual orientation of the heme and low spin cytochrome *f* with an α-band peak at 552 nm. Both hemes have significantly lower redox potentials than wild type cytochrome *f*. We attribute the high spin form to uncleaved pre-holocytochrome *f* and the low spin form to misprocessed forms of cytochrome *f* that were cleaved at a position different from the regular Ala^29*-Gln-Ala^31* motif. In contrast to F29L-31L, F32P displayed a small population of functional cytochrome *f*, presumably cleaved at Ala^29*, with characteristics close to those of wild type cytochrome *f*. The latter form would account for cytochrome *b*/*f* turnover and photosynthetic electron transport that sustain phototrophic growth of F32P.

*bc* complexes, cytochrome *bc*~1~ and cytochrome *b*/*f*, are electron-driven proton pumps that are present in the periplasmic bacterial membranes and in the inner membranes of mitochondria and chloroplasts. They oxidize lipophilic quinols and reduce water-soluble proteins, either c-type cytochromes or plastocyanin. In chloroplasts, plastocyanin oxidation occurs at a quinol-binding site of the protein complex located close to the luminal side of the thylakoid membrane. A first electron is transferred from the plastocyanin to a high potential chain, via a [2Fe-2S] center and a c-type cytochrome to the soluble acceptor, plastocyanin or cytochrome *c*~6~. A second electron is transferred across the membrane from the plastosemiquinone produced by the first oxidation step via two b hemes to a plastolignone bound at a quinone-reducing site of the complex close to the stromal side of the membrane. During the functional turnover of cytochrome *b*/*f* complex, there is a net release of protons at the luminal side of the membrane, whereas protons are taken up from the stromal side.

The cytochrome *b*/*f* complex from the unicellular green alga *Chlamydomonas reinhardtii* is similar to those from higher plants. It comprises at least seven subunits, five of which are chloroplast-encoded and two are nucleus-encoded (for a review see Ref. 3). The nuclear gene *petC* encodes the Rieske [2Fe2S] protein, which binds an iron sulfur cluster. The chloroplast genes *petA*, *petB*, and *petD* encode three major subunits of the complex, i.e. cytochrome *f*, cytochrome *b*~6~, and subunit IV, respectively. Cytochrome *b*~6~ binds two b hemes and cytochrome *f* a c-type heme. Subunit IV, as well as the three additional 3-kDa subunits (two chloroplast and one nuclear encoded), do not have cofactors.

Maturation of cytochrome *f* has been studied in some detail (reviewed in Ref. 3). Before its assembly into cytochrome *b*/*f* complexes, cytochrome *f* is inserted in the thylakoid membrane most likely by a co-translational process. It is converted from a pre-apoprotein into a mature holoprotein by two major maturation processes as follows: the cleavage of the transmembrane luminal targeting domain, a N-terminal extension of the polypeptide sequence, and the ligation of the heme. Both processes occur at the lumen side of the membrane. Genetic approaches using combined conventional and site-directed mutagenesis of *C. reinhardtii* have identified several nuclear-encoded factors and one chloroplast-encoded factor, which are required either for protein translocation (4) or heme ligation (5–8). The thylakoid processing peptidase (TPP), 1 homologous to the bacterial peptidase involved in the processing of secreted proteins (9), cleaves the presequence of cytochrome *f* right after a characteristic Ala-Gln-Ala motif. Tyrosine 32 thus becomes the N terminus of the mature protein (10, 11). Mature cytochrome *f* remains anchored in the membrane by its C-terminal α-helix (11, 12). The three-dimensional structure of the soluble part of cytochrome *f* revealed that the free amino group of the N-terminal tyrosine of the mature protein acts as sixth ligand to
the c heme (13). This unexpected observation suggested that conversion of pre-apocytochrome f to holocytocrome f requires cleavage of the trans peptide before heme ligation occurs. However, replacement of the cleavage site Ala-Gln-Ala by Leu-Gln-Leu allowed the precursor form of cytochrome f to covalently ligate a heme (2). This mutant, F29L-31L, was still able to assemble cytochrome b$_f$f complexes that comprised the precursor form as well as some slowly processed cytochrome f forms, but the strain could not grow under phototrophic conditions. Another site-directed mutant, Y1P (1), originally claimed to be a substitution of the N-terminal tyrosine by a proline, aimed at preventing formation of the sixth ligand to the heme, was shown not to assemble cytochrome b$_f$f complexes and therefore not to grow in phototrophic conditions. Further analysis of this mutant showed that the petA sequence bore an additional mutation that also altered the Cys-X-Y-Cys-His$_{56}$ critical c heme-binding motif, which is conserved in all c-type cytochromes; His$_{56}$ was converted to phenylalanine.5 Here we show that a bona fide Tyr$_{232}$/Pro substitution does not prevent phototrophic growth although it alters the processing of cytochrome f. We provide a comparative functional analysis of the phototrophic F32P mutant and the non-phototrophic F29L-31L mutant, which are both altered in cytochrome f processing.

### MATERIALS AND METHODS

**Cell Growth Condition**—The wild type strain of C. reinhardtii (mt + ) derived from the strain 157C and the chloroplast transformants were grown on Tris/HCl acetate/phosphate (TAP) and minimum media, pH 7.2, at 25 °C under 6 and 60 μM m$^{-2}$ s$^{-1}$ of continuous illumination, respectively. Y1P was obtained by courtesy of R. Malkin (Berkeley, CA). F29L-31L (2) and F32P were obtained in our laboratory (see below).

**Mutagenesis and Plasmids**—Mutagenesis was carried out on plasmid pWF (14) which encompasses the whole peta-coding region and its flanking sequences. Mutation of tyrosine 32 into proline was introduced by site-directed mutagenesis according to the method of Kunkel (15). Conversion of pWF to pF32P was obtained using the following mutagenic oligonucleotide: 5’-CTGAAAGCGGCTACGGATGCTTTACCCTGTA-3’. Amplification was performed in Escherichia coli, and screening of candidate colonies was carried out using the newly created NarI restriction site. Plasmid pIWF32P was constructed by introducing the 2-sulfonate fragment of plasmid pUC-atpX-AAD into the 1.9-kilobase pair aadA cassette (16) in the same orientation as the peta gene in the EcoRV site of plasmid pF32P.

**Chloroplast Transformation in C. reinhardtii**—The wild type and FBE mutant, which harbors a deletion of the peta gene (2), were transformed by tungsten particle bombardment (17). The particle gun was built in our laboratory by P. Bennoun and D. Be`al and is operating at 1300 mEm with a home-built spectrophotometer (28), with modifications (29) in the experiment. Intensity of one photon every 2 ms.

**Protein Isolation, Separation, and Analysis**—Membranes were prepared according to Chua and Bennoun (18) with modifications as in Atteia et al. (19). Polyphenols were separated by denaturing SDS-PAGE in the Laemmli system (20) using 12 and 12–18% acrylamide gels in the presence or absence of 8 M urea. Heme staining with TMBZ was performed according to Thomas et al. (21). Immunobots with specific antibodies against cytochrome b$_f$f complex subunits were used for protein analyses (14).

**Pulse Labeling Experiments**—Whole cells, grown to a density of 2 × 10$^{6}$ cells/ml, were concentrated 10 times and labeled for 5 and 45 min with [14C]acetate at the final concentration of 5 μCi/ml in the presence of 8 μg/ml cycloheximide as described in Delepelaire (22). The labeling reaction was stopped by addition of 1 volume of chilled 50 mM sodium acetate. Cells were harvested by centrifugation, resuspended in 100 mM dithiothreitol and 100 mM Na$_2$CO$_3$, and incubated in presence of 2% SDS at 100 °C for 1 min.

**Cytochrome b$_f$f Preparations**—Thylakoid membranes were solubilized in the presence of 25 mM hecagem (Vegetac, France) and loaded on a 10–35% sucrose gradient (23). After centrifugation, fractions were collected and subjected to SDS-PAGE.

**In Vitro Synthesis of Precurso and Mature Cytochrome f**—DNA fragments coding for the mature and precursor forms of cytochrome f were amplified with the following oligonucleotides: Cytf-3SR, 3’-GCCG-GAAATTCCCTTAAGATTTCTGTGATATGAA-5’; Cytf-matSR, 5’-GCCGGTACCAAAATATGCGACGTCAGTTATCCTGGA-3’, Cytf-preSR, 5’-GCCGGTACCAAAATATGCGACGTCAGTTATCCTGGA-3’. These clones were obtained using the plasmid pdWF as a template (14). Letters underlined indicate the restriction site KpnI in the oligonucleotide Cytf-3SR and the restriction site EcoRV in Cytf-matSR and Cytf-preSR. The fragments corresponding to the coding sequence of the mature and precursor protein were cloned in the pBSK plasmid and cut with KpnI and EcoRI, thus leading to plasmids pMatSR and pPreSR, respectively. These two plasmids were used as templates to perform in vitro transcription using the Promega Kit, according to the manufacturer’s protocol. The resulting RNAs were translated in wheat germ system (Amersham Pharma
cia Biotech) in the presence of [35S]methionine (24).

**Electrochemical Titration on Membrane Fragments**—Membranes were prepared from a double mutant without PSII (obtained by crossing our mutants with the PSII-less mutant strain F35 (25)) to allow titration of the α-bands of the cytochrome b$_f$f complex in the absence of signals from cytochrome b$_{op}$. The membrane pellet was resuspended in 1 volume of 1 M MOOPS buffer, pH 7, 0.1 M KCl, in the presence of a mixture of 12 mediators (Table 1) at a concentration of 2 × 10$^{-4}$ M each. Final concentration of mediators was 10$^{-4}$ M. This solution was incubated for 30 min on ice before filling of the electrochemical cell (26, 27). The gold mesh, which was used as a working electrode in the cell, was modified with pyridine-3-carboxaldehydehethiosemicarbazon in order to avoid irreversible protein adhesion. Experiments were done at 12 °C. Titrations in steps of 30 mV were performed in the oxidative and the reductive directions in the potential range from +450 to −500 mV. Equilibration time was between 5 and 12 min. Spectra were recorded with a home-built spectrophotometer (28), with modifications (29) in the spectrum modus. The titration data were evaluated by the global fit procedure mEh-fit (30) in order to obtain redox potential and amplitude of each redox-active and -absorbing compound.

**Kinetic Analysis in Vivo**—Algae were centrifuged and resuspended in 50 mM MOOPS buffer, pH 7, with 10% Ficoll in order to slow down the sedimentation of the algae in the cuvette. The resuspended algae were oxygenated at low light for 1 h by intense shaking before the start of the experiment. 10$^{-3}$ M DCMU and 10$^{-3}$ M HA were added to inhibit photosystem II (31). The algae were kept in anaerobiosis under a stream of argon in the cuvette. FCCP was added at a concentration of 0.5–2 × 10$^{-6}$ M to collapse the permanent transmembrane electrochemical potential. Electron transfer reactions were triggered by an actinic flash from a Xenon lamp, filtered by a dark red filter (RG695). Each flash excited about 20% of PSI centers. The interval between actinic flashes was 8 s in our experiments. Continuous light was used at an intensity of one photon every 2 ms.

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2 F. Zito and F.-A. Wollman, unpublished observations.
Mutants of Cytochrome f Maturation

FIG. 1. Heme stain and immunoblot analysis of cytochrome f. A, immunoblot of whole cell protein extract from the wild type (wt), F32P, and ΔpetA strains, probed with a specific antisera against cytochrome (cyt)f, and revealed using 125I-labeled protein A (Amersham Pharmacia Biotech). SDS-PAGE was performed with whole cell protein extracts loaded at constant chlorophyll (20 μg/ml) on a 12–18% acrylamide gel containing 8 M urea. Cytochrome f antisera was diluted at 1/100. B, immunoblot performed with antisera against cytochrome f, cytochrome b6, and OEE3 after whole cell protein separation by SDS-PAGE using a 12–18% acrylamide gradient. OEE3 was used as loading control. C, heme staining of cytochrome f in F32P. Electrophoretic conditions as in B. Whole cell protein extracts were loaded at constant chlorophyll (20 μg/ml) on a SDS-PAGE (12–18% acrylamide). Arrows indicate the three forms of cytochrome f in the mutant.

**EPR Analysis**—EPR spectra were recorded on membrane fragments of mutants and wild type using an EFS 300 X-band spectrometer fitted with an Oxford Instruments He cryostat and a temperature control system. Since the membranes were oxidized upon preparation, reduction of cytochrome f and the Rieske protein was achieved by washing the samples with 5 mM ascorbate. 20 μM dithionite was added in order to reduce the plastoquinone pool. Washing with 2 mM ferricyanide oxidized all redox centers of the b_{f} complex. EPR redox titrations were performed according to Dutton (32) using the mediator combination indicated in Table I at a concentration of 10^{-4} M. For the study of oriented samples either in their oxidized state or in their reduced state when exposed to 5 mM ascorbate, membranes were washed twice with distilled water. Resuspended membranes were applied on Mylar sheets and dried in a humidity-controlled atmosphere under argon for several days (33).

**Fluorescence Measurements**—Fluorescence measurements were performed with a home-built fluorimeter. The continuous actinic light, provided by electroluminescent diodes, was set at 590 nm and at an intensity of 60 μW s^{-2} m^{-2}. Fluorescence was measured at right angles with photodiodes UV444B. F_{max} levels were determined in the presence of 10^{-5} M DCMU.

**RESULTS**

We have analyzed the cytochrome f content in two site-directed *petA* mutants aimed at altering the conversion of pre-apocytochrome f to holocytochrome f. F32P has a proline at the place of the N-terminal tyrosine 32 which serves as the sixth ligand to the heme. Proline is the only amino acid unable to serve as a ligand to the heme due to the absence of an N-terminal amino group. Tyrosine 32 is situated immediately downstream of the recognition site for the TPP.

**Protein Analysis**—After polypeptide separation by SDS-PAGE, both with and without urea, three forms of cytochrome f were observed in F32P (Fig. 1, A and B). These should be compared with the mature form present in the wild type and with absence of a cytochrome f band in a strain deleted for the *petA* gene. We rule out that the three bands may reflect different states of denaturation of the same protein since we found the same triplet of bands under stronger denaturing conditions, *i.e.*, 0.5% instead of 0.1% SDS in the running buffer (data not shown). The three forms were found at the same positions as those previously reported for F29L-31L, which bears a Leu-Gln-Leu motif instead of the regular Ala-Gln-Ala motif, thereby hampering the action of TPP (2). The three forms of cytochrome f, which were detected with a specific antibody (Fig. 1, A and B), were also detected by TMBZ staining which identifies mainly heme-containing polypeptides (Fig. 1C). Thus, the three forms of cytochrome f in F32P are heme-binding polypeptides in agreement with our previous report of F29L-31L (2).

The steady state proportions of the three forms of cytochrome f differed between the two mutants. In F29L-31L the lower band was the most abundant (2), whereas in F32P the lower form is the less represented when algae were grown heterotropically. However, when F32P was grown under phototrophic conditions, the shortest form of cytochrome f became the prominent form (data not shown).

**Comparison to in Vivo Synthesized Apo- and Pre-apocytochrome f**—In order to compare the migration positions of the three bands of mutated cytochrome f with the precursor and mature apoforms of wild type cytochrome f, the latter were expressed in *vitro* and radiolabeled with [35S]methionine using cloned petA genes containing or not the presence of cytochrome f (see “Materials and Methods”). The electrophoretic migration positions of the precursor and mature polypeptide were compared with those of cytochrome f as detected upon 5- and 45-min pulse labeling with [14C]acetate of cells from the wild type, F32P, F29L-31L, and Y1P (Fig. 2, A–C). The mature form of cytochrome f expressed in *vitro* (Fig. 2C) migrated at a position similar to that of cytochrome f expressed in vivo in the wild type. The precursor form of cytochrome f, as detected when expressed in *vitro*, migrated at a position higher than the mature form. In 5-min pulses, F32P and F29L-31L displayed a cytochrome f doublet (1 and 1*) in Fig. 2) at this position. The lower band (*) in Fig. 2, A and C) was absent in 45-min pulses of the wild type, F32P, and F29L-31L. As we had shown previously for F29L-31L (2), pre-apocytochrome f is converted within a few minutes in some holoform. Thus we assigned band 1* to pre-apocytochrome f and band 1, the upper band of the doublet, to pre-holocytochrome f. Consistent with this latter attribution, pre-holocytochrome f was long-lived enough to be detected in 45-min pulses (Fig. 2B) as well as by conventional detection procedures (upper band of the triplet in Fig. 1). Additionally, two forms of lower apparent molecular weight appeared in 45-min pulses with the mutants. Their electrophoretic migration positions corresponded to that of the two lower bands observed by conventional detection procedures (Fig. 1). The lower of these two bands migrated at a position similar to that of mature cytochrome f (Fig. 2, A and B).

Y1P is unable to bind heme, due to the absence of His^{56}_{e}, the fifth ligand of the c heme. Therefore, pre-apocytochrome f (band 1*) appeared in Y1P in 5-min pulses and was retained in 45-min pulses, because of its inability to undergo an apo- to holoconversion.

**Complex Assembly**—The steady state accumulation of one precursor and several processed forms of cytochrome f raised the question of their actual assembly into cytochrome b_{f} complexes. To answer this point, thylakoid membranes from F32P were solubilized according to Pierre et al. (23, 34), and cytochrome b_{f} complexes were recovered from the supernatant upon sucrose gradient centrifugation. The three forms of cytochrome f were found in the gradient fractions that also contained cytochrome b_{6} (Fig. 3). Thus the precursor as well as the processed forms of cytochrome f assembled into cytochrome b_{f} complexes. In addition these protein complexes showed a bimodal distribution along the sucrose gradient. The population of mutated protein complexes of lower buoyant density was enriched in the processed forms of cytochrome f and migrated at the same position as the genuine dimeric cytochrome b_{f} com-
in vitro labeled pulse labeling of wild type (fs, sor forms of cytochrome F29L-31L, and Y1P). In contrast, the kinetics than in the presence of the inhibitor. This is indicative of a lower efficiency of plastoquinol re-oxidation. Table II shows a quantification of this LEF index for the four strains shown in Fig. 4. Also shown in Table II is the quenching at $F_{\text{max}}$ observed when the cells were transferred from the conditions used in Fig. 4, i.e. strongly oxygenated by vigorous stirring in the darkness, to anaerobic conditions. This switch promotes transition from state I to state II in the wild type, with a typical quenching at $F_{\text{max}}$ of about 40–50% (35, 36). This transition requires the presence of a bound quinol at the Qo site of cytochrome $b_{\text{f}}$ complexes (37, 49). Thus, a mutant lacking cytochrome $b_{\text{f}}$ complexes does not undergo state transitions (Table II). F32P developed a transition to state II in anaerobic conditions but of smaller amplitude than the wild type. We detected a very small but reproducible quenching under anaerobic conditions with F29L-31L. The interaction of a quinol or quinone with the Qo site can be detected by its influence on the $g_x$ signal of the reduced [2Fe2S] protein. As shown in Fig. 5, the position and shape of this signal revealed significant differences between the wild type and the mutants. The [2Fe2S] protein of the wild type had a relatively narrow $g_x$ signal at 1.76 when the plastoquinone pool was in its reduced state (dithionite-reduced samples, thin lines in Fig. 5). Upon oxidation of the plastoquinone pool (ascorbate-reduced samples, bold lines in Fig. 5), the signal became broader and shifted to $g = 1.73$. The $g_x$ signal of F29L-31L is relatively narrow, irrespective of the redox state of the plastoquinone pool. However, a slight shift of this signal was still observed; it was at $g = 1.75$ when the pool was oxidized but at $g = 1.77$ when the pool was reduced. F32P displayed a broad $g_x$ trough in both redox states of the plastoquinone pool, with a minor contribution of a wild type signal (see arrows on Fig. 5).

It should be noted that a large signal at $g = 1.92$ was observed in dithionite-reduced samples from the wild type and mutants. This signal is reminiscent of those observed by Nu-
In Vivo Measurements of Electron Transfer Kinetics—We studied the kinetics of electron transfer through the cytochrome b₆f complex by single turnover flash spectroscopy using algae placed under anaerobic conditions (Fig. 6). Photosystem II photochemistry was blocked by addition of HA and DCMU. Thus illumination by a flash produced charge separation only in photosystem I. Consequently plastocyanin was oxidized by P₇₀₀⁺, subsequently oxidizing cytochrome f, thus triggering cytochrome b₆f turnover. Flashes of low intensity (i.e. 20% of a saturating flash) were chosen in order to produce less than one oxidized plastocyanin per cytochrome b₆f complex in the wild type, therefore avoiding double turnovers of the cytochrome b₆f complex. Fig. 6 displays the 515 nm absorbance changes observed after one flash. The 515 nm signal corresponds to a carotenoid band shift that develops when the transmembrane potential changes (29, 40). After one flash there was an early rise, below the time resolution of our spectrophotometer, that corresponds to the charge separation in PSI, followed by a millisecond rise phase that corresponds to a cytochrome b₆f complex-mediated charge translocation across the membrane. The half-time of the slow phase is sensitive to the presence of a permanent electrochemical transmembrane gradient in the living algae. This gradient can be collapsed by addition of FCCP. In agreement with previous reports (41), the wild type showed a half-time of 5–7 ms in the presence and 1–3 ms in the absence of a permanent transmembrane potential. The slow phase of the 515 nm signal was absent in F₂₉L-₃₁L under all conditions tested, whereas F₃₂P displayed a half-time of 50–70 ms versus 20 ms in the presence or absence of a permanent transmembrane potential. We noted that several hours of incubation in anaerobic conditions were required for F₃₂P to develop the slow phase of the carotenoid band shift. This was in marked contrast to the wild type that displayed the slow phase as soon as anaerobic conditions were established.

When F₃₂P was placed under continuous illumination or subjected to a series of saturating flashes, it accumulated oxidized cytochrome f because cytochrome f reduction was rate-
limiting under these conditions (Fig. 7 middle). In contrast, F29L-31L accumulated oxidized plastocyanin (Fig. 7, bottom panel) and P700 (not shown), but we could not detect any significant amount of oxidized cytochrome f. In Fig. 7 (top panel) a flash-induced spectrum of cytochrome f from the wild type is shown for comparison. It is noteworthy that the a-band peak of cytochrome f in F32P was of small amplitude, slightly broader, but in the same spectral position at 554 nm as in the wild type.

**Redox Titrations**—In order to detect all spectral forms of cytochrome f, independent of their participation in photosynthetic electron transport, we performed redox titrations of the a-band peak of cytochrome f and cytochromes b using a membrane fraction isolated from the three strains. Since absorption signals from cytochrome b563 associated with PS II prevented us from observing small spectral changes induced by subpopulations of cytochrome b6f complexes with altered properties of cytochrome f, we resorted to the use of mutants that lacked PSII. We used the mutant F35 instead of the wild type, as a control, and we generated double mutants F29L-31L*F35 and F32P*F35 by genetic crosses between the F35 nuclear mutant (mt^2) and the site-directed petA chloroplast mutants (mt^1). A midpoint potential of +350 mV and an a-band peak at 554 nm were obtained for cytochrome f from F35 mutant (Fig. 8). The Em values for the b hemes were found at -70 and -170 mV, respectively. Redox titrations with the wild type yielded similar values (results not shown). The amplitude of the peak for cytochrome f in the wild type and F35 mutant corresponded to the amplitude of one of the b hemes. For both F29L-31L*F35 and F32P*F35 we found a low spin cytochrome f with an a-band peak at 552 nm. Its amplitude was only about 1/4 of the amplitude of one of the b hemes, and the midpoint potential was at +120/-140 mV, which is about 220 mV more negative than cytochrome f of the wild type. The redox properties of the b hemes were not affected in the mutants. F32P*F35 displayed an additional low spin cytochrome f. It was reduced at +260 mV and oxidized at +450 mV and had an a-band peak at 554

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**Fig. 5. EPR spectra of the Rieske signals.** EPR spectra of membrane fragments of wild type (WT), F32P (Pro), and F29L-31L (Pen) reduced by ascorbate, i.e. with oxidized plastoquinone pool (thin line) and with dithionite-reduced plastoquinone pool (bold line). The g_s signal of the Rieske protein for wild type and mutants is at g = 1.89 g. The g value of the g_s trough depends on the redox state of the plastoquinone pool and differs between wild type and mutants. The signal at g = 1.92, which appears in the dithionite-reduced samples, will be described in detail elsewhere. The two insets at the right give a closer, comparative view to the g_s signals. EPR parameter were as follows: temperature 15 K, microwave power 6.7 milliwatts, microwave frequency 9.42 GHz, modulation amplitude 32 G.

**Fig. 6. Kinetics of the b6f complexes.** Electrochromic shift of carotenoids as induced by changes in the electrochemical transmembrane potential, followed at 515 nm. Samples were treated with 0.1 μM FCCP, 10^-3 mM HA, and 10^-5 mM DCMU. The rapid phase is beyond the resolution of our spectrophotometer and corresponds to charge separation in photosystem I. The slow phase corresponds to charge transfer in the b6f complex. It was corrected for the potential decline due to charge equilibration across the membrane and fitted to an exponential.

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4 All redox potentials are given versus the standard hydrogen electrode.
nm (Fig. 9). We could not perform a redox titrations of this peak due to its small amplitude. It corresponded to less than 1/10 of the amplitude of one b heme and was slightly broader than the \(\alpha\)-band peak of cytochrome \(f\) from the wild type. We tentatively assigned the other peak at 550 nm, present in both mutants, to cytochrome \(c_1\) from mitochondrial \(bc_1\)-complex since mitochondrial membranes are present in our thylakoid membrane preparations (19).

**EPR Investigations of High Spin Cytochromes**—We also looked for the presence of high spin forms of cytochrome \(f\) in the mutants, because we have altered the sixth ligand of the \(c\) heme in F32P, and the above-detected cytochrome \(f\) signals accounted only for 30–40% of cytochrome \(f\). To this end we performed EPR experiments using membrane preparations from each strain. The low spin EPR signal for cytochrome \(f\), at \(g = 2.0\), was not detectable in the mutants (Fig. 10). Fig. 11 shows the normalized high spin signals at around \(g = 6\) for wild type and mutants. High spin signals were already present in membrane preparations from the wild type. They most likely originated from denatured b hemes in the membrane, and their amount was variable from one preparation to the other. The \(g = 6\) signal of the wild type showed a rhombic distortion into distinguishable \(g_x\) and \(g_y\) lines. In the mutants this feature was overlapped by a signal of higher axial symmetry as evidenced by a lower relative contribution of the high field shoulder. Experiments on partially ordered membranes were undertaken to investigate further the orientation of these signals. The amplitudes of the \(g = 6\) signals, which represent the \(g_x\) and the \(g_y\) of high spin hemes, were plotted against the angle between the membrane plane and the magnetic field. The resulting polar plots are shown in Fig. 12. The wild type signal showed an orientation at 45 °C in the ascorbate-reduced state and at 45, 0, and 90 °C in the oxidized state. Because heme signals can be observed by EPR only when the heme is in its oxidized state, these signals indicated the presence of at least two different high spin hemes in the wild type membranes. One

**FIG. 7. Light-induced spectra.** Top, spectrum of wild type (WT) cytochrome \(f\) (cyt \(f\)) obtained 100 \(\mu s\) after a saturating flash; middle, spectrum of F32P taken 500 \(\mu s\) after 10 saturating flashes at a 20-ms interval; bottom, spectrum of F29L-31L taken after continuous illumination with one photon every 2 ms. The spectrum of oxidized plastocyanin obtained for F29L-31L is stable for several seconds after the end of illumination. Amplitudes of all spectra were normalized to the same Photosystem I concentration, as measured by the amplitude of the rapid phase of 515 nm absorbance (Abs.) change. r.u., relative units.

**FIG. 8. Redox titration of cytochrome \(f\) complex.** Redox titrations using membrane fragments from wild type (left panel), F29L-31L (right panel), and F32P (middle panel). Potential titrations was performed in steps of 30 mV in the potential range from +480 to −400 mV. Spectra were recorded at each potential and evaluated by a global fit.
population is reducible by ascorbate and the other one is not. We tentatively attribute these signals to denatured forms of cytochrome $b_{559}$ and cytochrome $b_{6}$, respectively.

The $g_x$ and $g_y$ directions of high spin hemes are parallel to the heme plane and perpendicular to each other. If $g_x$ and $g_y$ are both at 45 °C, the heme is therefore oriented perpendicular to the membrane. This signal was observed in the reduced state and is still present in the oxidized state, where two additional orientations appeared at 0 and 90 °C. The latter orientation corresponds to a heme, again perpendicular to the membrane, and the orientations confirm our assignment to cytochrome $b_{559}$ and $b_{6}$. With the partially ordered membranes of the mutants, additional signals were present in the ascorbate-reduced and in the oxidized state. $g_x$ and $g_y$ were at about 15 and 70 °C (arrows in Fig. 12). The corresponding heme therefore had a midpoint potential below +100 mV and was oriented close to 80 °C with respect to the membrane plane. Thus the EPR analysis of the $g = 6$ region showed that an additional high spin heme was present in the cytochrome $f$ mutants. Its orientation at about 80 °C should be compared with that at 30 °C for low spin cytochrome $f$ in the wild type (see Footnote 4 for cytochrome $f$ of C. reinhardtii and Refs. 42 and 43 for cytochrome $f$ of higher plants).

**DISCUSSION**

The thylakoid processing peptidase that converts pre-cytochrome $f$ to its mature form recognizes a typical Ala-X-Ala motif, similar to its bacterial homologue for secretory proteins. 151 cleavage sites of proteins from Gram-negative bacteria were investigated, and none has a proline residue in position...
+1, immediately downstream the cleavage site. Introduction of a proline in the +1 position of secreted proteins in *E. coli* prevents protein processing *in vivo* (44–47). In this study we show that placing a proline residue in that position in *Chlamydomonas* hampers protein processing by the TPP in a very similar way as the replacement of the Ala-Gln-Ala processing motif by Leu-Gln-Leu. Substitutions at the +1 position by other residues than a proline did not alter cytochrome f processing (1).

In *E. coli* proteins, introduction of a proline at position +1 created a signal peptide that binds to the processing peptidase and can no longer be cleaved. The protein therefore stands as an uncleavable substrate associated with the enzyme (45, 46). The two processing mutants of cytochrome f, F32P and F29L-31L, exhibited a light and a heavy form of cytochrome b$_6$f complexes that can be separated upon sucrose gradient centrifugation. We noted that the unprocessed and intermediate forms of cytochrome f accumulated in a population of higher buoyant density than those of regular cytochrome b$_6$f dimers. We therefore assume that this heavy form may reflect a physical association of the cytochrome b$_6$f complexes containing pre-cytochrome f with the TPP. Further biochemical analysis of these gradient fractions is in progress to identify possible TPP candidates.

Both mutants displayed a slow processing of the precursor form of cytochrome f. In addition, the precursor form as well as the processed forms bound hemes and assembled into cytochrome b$_6$f complexes. These assemblies were stable in the membranes since their steady state concentration, as probed by the content in cytochrome b$_6$ and subunit IV relative to the content in photosystem II subunits OEE2 and OEE3, remained similar to that in the wild type. Thus the behavior of F32P confirmed our previous report that cytochrome f processing is not a prerequisite for heme binding or cytochrome b$_6$f assembly in *C. reinhardtii* (2).

Fluorescence measurements and investigations of the changes in transmembrane potential showed no evidence for photosynthetic electron flow through the cytochrome b$_6$f complexes of F29L-31L, thus explaining why the mutant could not grow phototrophically. The forms of cytochrome f detected in this mutant should then contribute only inactive cytochrome b$_6$f complexes. One of these forms, also present in F32P, was a low spin cytochrome f, i.e. a cytochrome that still has two axial ligands to the heme. Its redox potential and spectral characteristics were distinct from those observed in the wild type. This form would account for about 30% of the total population of cytochrome b$_6$f complexes in both mutants. It displayed an a-band peak at 552 nm, and its midpoint potential was shifted by 200 mV to more reducing potentials than cytochrome f from the wild type. As we demonstrated here using F29L-F31L, this

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**Fig. 11. EPR spectra of the g = 6 signal.** EPR spectra of oxidized membrane fragments show a rhombic distortion into distinguishable $g_x$ and $g_y$ lines for the $g = 6$ signal of the wild type (WT). In the mutants this feature was overlapped by a signal of higher axial symmetry as evidenced by a lower relative contribution of the high field shoulder, parameters as in Fig. 5.

**Fig. 12. Polar plots of the g = 6 signal.** Two high spin hemes, with an orientation perpendicular to the membrane, are present in the wild type (WT). One of these hemes, presenting the $g_x$ and $g_y$ orientations at 0 and 90 °C, is reducible by ascorbate (solid line), and the other one with $g_x$ and $g_y$ orientations at 45 °C is not. In the mutants additional orientations are present (arrows) at about 15 and 70 °C, referring to an additional high spin heme with an orientation of about 80 °C with respect to the membrane plane.
Mutants of Cytochrome f Maturation

Fig. 13. Schematic representation of putative alternative processing sites in cytochrome f mutants. Hypothetical cleavage sites and resulting heme ligation in wild type, F32P, and F29L-31L are illustrated. The diamond represents the heme. The N terminus of the protein and other putative ligands to the heme are indicated by circles; arrows point to the putative alternative cleavage sites; a, cleavage at Ala29 of wild type cytochrome f; p1, cleavage at Ala29, only possible in F32P, resulting in a functional protein contributing to the band 3 on a gel; p2 and p3, cleavage at Ala29 (band 3 on gel) or Ala62 (band 2 on gel), for both mutants, leading to non-functional low spin hemes; p4, uncleaved pre-holocytochrome f with the N-terminal transmembrane helix and a high spin heme, missing the sixth ligand (band 1 on gel). On the bottom of the figure the N-terminal sequence of wild type cytochrome f is presented. The Ala29-Gln-Ala31 motif mutated to Leu-Gln-Leu in F29L-31L is underlined by dots; Tyr32, mutated to Pro in F32P, is underlined by dashes.

Form is inactive in electron transfer since it does not undergo any light-induced redox changes. A high spin form of cytochrome f, which would have only His66 as a fifth ligand, was also identified in both mutants. The high axial symmetry of the high spin heme (Fig. 11) may originate from a water molecule in place of the sixth ligand. Its orientation was at about 80 °C with respect to the membrane plane, and the midpoint potential was below +100 mV. This unusual orientation, i.e. cytochrome f of the wild type is tilted by 30 °C with respect to the membrane, may prevent electron transfer from the [2Fe-2S] protein to the heme. The lower redox potential of the two forms of hemes above-described argues for their exposure to a more hydrophilic environment and supports the idea that electron transfer is inhibited between the [2Fe2S] cluster and these two forms of cytochrome f.

Despite the very similar biochemical characteristics of the two processing mutants, only did F32P grow phototrophically and display an additional low spin cytochrome f that is present in small amounts. It kept a high midpoint potential (between +260 mV and +450 mV) and the same visible spectrum (a-band at 554 nm) as cytochrome f in the wild type. We attribute the unique phototrophic properties of F32P to the presence of a functional protein contributing to the band 3 on a gel; p2 and p3, cleavage at Ala29 (band 3 on gel) or Ala62 (band 2 on gel), for both mutants, leading to non-functional low spin hemes; p4, uncleaved pre-holocytochrome f with the N-terminal transmembrane helix and a high spin heme, missing the sixth ligand (band 1 on gel). On the bottom of the figure the N-terminal sequence of wild type cytochrome f is presented. The Ala29-Gln-Ala31 motif mutated to Leu-Gln-Leu in F29L-31L is underlined by dots; Tyr32, mutated to Pro in F32P, is underlined by dashes.

The photosynthetically active cytochrome f, which would have only His66 as a fifth ligand, was also identified in both mutants. The high axial symmetry of the high spin heme (Fig. 11) may originate from a water molecule in place of the sixth ligand. Its orientation was at about 80 °C with respect to the membrane plane, and the midpoint potential was below +100 mV. This unusual orientation, i.e. cytochrome f of the wild type is tilted by 30 °C with respect to the membrane, may prevent electron transfer from the [2Fe-2S] protein to the heme. The lower redox potential of the two forms of hemes above-described argues for their exposure to a more hydrophilic environment and supports the idea that electron transfer is inhibited between the [2Fe2S] cluster and these two forms of cytochrome f.

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heme from the surrounding medium, and its position seems to be constrained mainly by the axial ligation of Tyr to the c heme. Besides this interaction, the peptide is not closely packed into the structure. In pre-cytochrome f this peptide is retained together with the presequence. The hydrophobic presequence has a transmembrane orientation, and an elongated helical motif with close to wild type properties. This active form of cytochrome f, which was assigned to band 1 on gels, corresponds to the high spin form of the heme with the low midpoint potential that we observed for both mutants.

An alanine residue in position –1 and the absence of a glycine in position –3 from the putative processing site are critical requirements for the action of the peptidase (47). Processing should therefore be possible only at Ala15 and Ala36 in both mutants, preventing formation of the short α-helix above-described or leaving numerous hydrophobic residues exposed to the luminal space if processing occurred at Ala15. In the two cases, the N terminus of the protein, and also Met20 in case of processing at Ala15, could serve as a sixth ligand to the heme. Abnormal processing will result in an altered protein structure that may enhance heme exposure to the hydrophilic environment, resulting in a shift of the midpoint potential to more negative values. Therefore, these forms may correspond to the precursor form of holo-cytochrome f, that may enhance heme exposure to the hydrophilic environment.

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