Maturation of Pre-apocytochrome f in Vivo

A SITE-DIRECTED MUTAGENESIS STUDY IN CHLAMYDOMONAS REINHARDTII*

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The biosynthesis of cytochrome f is a multistep process which requires processing of the precursor protein and covalent ligation of a c-heme upon membrane insertion of the protein. The crystal structure of a soluble form of cytochrome f has revealed that one axial ligand of the c-heme is provided by the α-amino group of Tyr1 generated upon cleavage of the signal sequence from the precursor protein (Martinez S. E., Huang D., Szczepaniak A., Cramer W. A., and Smith J. L. (1994) Structure 2, 95–105). We therefore investigated, by site-directed mutagenesis, the possible interplay between protein processing and heme attachment to cytochrome f in Chlamydomonas reinhardtii. These modifications were performed by chloroplast transformation using a petA gene encoding the full-length precursor protein and also a truncated version lacking the C-terminal membrane anchor. We first substituted the two cysteinyl residues responsible for covalent ligation of the c-heme, by a valine and a leucine, and showed that heme binding is not a prerequisite for cytochrome f processing. In another series of experiments, we replaced the consensus cleavage site for the thylakoid processing peptidase, AQA, by an LQL sequence. The resulting transformants were nonphototropic and displayed delayed processing of the precursor form of cytochrome f, but nonetheless both the precursor and processed forms showed heme binding and assembled in cytochrome b$_{6}$f complexes. Thus, pre-apocytochrome f adopts a suitable conformation for the cysteinyl residues to be substrates of the heme lyase and pre-holocytochrome f folds in an assembly-competent conformation. In the last series of experiments, we compared the rates of synthesis and degradation of the various forms of cytochrome f in the four types of transformants under study: (i) the C terminus membrane anchor apparently down-regulates the rate of synthesis of cytochrome f and (ii) degradation of misfolded forms of cytochrome f occurs by a proteolytic system intimately associated with the thylakoid membranes.

The presence of quinol-oxidizing proteins is a common feature of energy transducing membranes. In most cases, these transmembrane proteins are multisubunit protein complexes with similar characteristics in bacteria and mitochondria (cytochrome b$_{6}$c$_{1}$ complexes) and in chloroplasts (cytochrome b$_{6}$f complexes). Their activity results from intraprotein electron transfer reactions between an Fe-S center, two b-hemes and one c-heme, which couple translocation of protons across the membranes to oxidation of lipophilic electron carriers, ubiquinols or plastoquinols, and reduction of small hydrophilic proteins, a c-type cytochrome or a copper-containing protein, plastocyanin (Cramer et al., 1991).

The assembly of such oligomeric proteins is a multistep process involving the biosynthesis and delivery of several prosthetic groups to the various subunits whose interactions are required for the function of the mature protein. One of the major subunits of cytochrome b$_{6}$f complex is a c-type cytochrome which is membrane-bound by a single hydrophobic α-helix located in the C-terminal domain of the protein (for reviews, see Gray (1992) and Howe and Merchant (1994)). Most of the protein, including its N terminus and the heme-binding domain, extends into an aqueous environment facing the membrane on the side opposite that where membrane insertion is initiated. The final orientation of the mature protein is achieved by the cleavage of the N-terminal extension of the precursor form. Therefore, the cleavable sequence acts as a signal sequence for the proper localization of the mature cytochrome.

The chloroplast protein, cytochrome f, is encoded by the petA gene located on the organelle genome. In Chlamydomonas reinhardtii, the petA gene is located upstream of the petD gene encoding subunit IV, another subunit of the cytochrome b$_{6}$f complex (Büschlen et al., 1991). Even though the two can be cotranscribed (Sturm et al., 1994), they are translated from monocistronic transcripts (Kuras and Wollman, 1994). The petA message is translated on polysomes which are bound to the thylakoid membranes in pea chloroplasts (Gray et al., 1984) as well as in C. reinhardtii.1

Conversion of the petA gene product to a photosynthetic protein fully competent for electron transport requires four major events: membrane insertion of the precursor protein, processing of the precursor form, heme attachment to the polypeptide chain, and assembly in cytochrome b$_{6}$f complexes. The temporal sequence of, and the possible interplay between, these four events remains largely unknown. However, several recent studies have provided new insights on the biogenesis of cytochrome f and enable further dissection of the biosynthesis pathway.

Membrane translocation of the precursor protein requires a recognition process of the N terminus presequence since several mutations in its hydrophobic core were shown to prevent membrane insertion and maturation of the protein (Smith and Kohorn, 1994). Upon membrane translocation, the transmembrane anchor next to the C terminus would act as a stop

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1 D. Drapier and F.-A. Wollman, unpublished observations.
Conversion of piWF into pF52L-55V was achieved using a 40-base segment results in the complete translocation of a fully processed and redox-active soluble cytochrome (Kuras and Wollman, 1995). We observed that the overall rate of synthesis of cytochrome f is an assembly-controlled process since the absence of either subunit IV or cytochrome b₆ resulted in decreased rates of synthesis of cytochrome f with no decrease in the lifetime of the holoprotein (Kuras and Wollman, 1994).

N-terminal processing of cytochrome f and covalent attachment of the c-heme to the conserved protein motif CXXCH would both occur on the lumen side of the thylakoid membranes (Howe and Merchant, 1994). Therefore, both steps require translocation of part of the polypeptide sequence across the membrane but nevertheless can occur in the absence of translation of the C terminus membrane anchor (Kuras et al., 1995).

The recent determination of the crystal structure at 2.3-Å resolution of the soluble domain of cytochrome f from turnip (Martinez et al., 1994) unexpectedly revealed that one axial ligand of the c-heme is provided by the α-amino group of Tyr⁴, the amino-terminal residue of the mature protein. This is in variance with the other cytochromes c whose axial ligands are usually provided by side chains from methionine residues within the polypeptide core. It was therefore suggested that heme attachment to cytochrome f requires, and is subsequent to, processing of the precursor protein (Cramer et al., 1994; Howe and Merchant, 1994).

In order to unravel the possible interactions between precursor maturation and heme ligation, we used chloroplast gene replacement in C. reinhardtii to perform site-specific modifications aimed at preventing heme attachment or precytocrome f processing. The biosynthetic consequences of such alterations were further examined on a truncated protein which lacked the membrane anchor in the C-terminal region.

MATERIALS AND METHODS

Cell Growth Conditions—Wild-type and transformant cells were grown on Tris-acetate-phosphate (TAP) medium (Harris, 1989), pH 7.2, at 25°C under continuous dim light (300 lux).

Mutagenesis and Plasmids—Oligonucleotide primers for mutagenesis were synthesized using an LKB DNA synthesizer and purified according to the manufacturer’s protocol. Site-directed mutagenesis was performed in Escherichia coli according to the method of Kunkel (1987). The mutated products were verified by sequencing. Mutagenesis were carried out on plasmid piWF (Kuras and Wollman, 1994) which encompasses the whole petA coding sequence and its flanking regions. Conversion of piWF into pF52L-55V was achieved using a 40-base oligonucleotide primer. The GTATGTCGCAAAGCTGACAC. This led to a neutral mutation for the Val51 codon, converted the Cys52 codon to a Lys codon linked to a new NheI restriction site and converted the Cys55 codon to a Val codon linked to a new SnaI restriction site. A 37-base oligonucleotide primer was used to convert piWF into pF52L-55V. The CCGCGGACGCTGACAC. This converted the Ala20 codon to a Lys codon linked to a new NheI restriction site and converted the Cys25 codon to a Lys codon linked to a new NheI restriction site and led to a neutral mutation for the Gin26 codon. Plasmids pF52L-31L and pF52L-55V were constructed by exchanging the 3′-XbaI fragment from both pF52L-31L and pF52L-55V with an equivalent fragment of pF1283T (Kuras et al., 1995). The Act-XbaI fragment encompasses the end of the petA coding region and its 3′-flanking region. Plasmid pF1283T bears a mutation which replaces the Ile283 codon of the petA gene by a stop codon. Plasmids pF52L-31L, pF52L-55V, pF52L-31L, pF52L-55V, and pF52L-55V were constructed, respectively, by introducing the 1.9-kilobase aadA cassette in the same orientation as the petA gene in the EcrV site of plasmids pF52L-31L, pF52L-55V, pF52L-31L, pF52L-55V, and pF52L-55V. The EcrV site is located 309 base pairs downstream from the petA coding region in the intergenic petA-petD region. The aadA cassette was obtained by Smal-EcoRV digestion of plasmid pUC-aphA (Goldschmidt-Clermont, 1991). Strains and Transformation—C. reinhardtii cells were transformed with a particle gun as described by Boynton et al. (1988). In a first attempt, we used plasmids pF29L-31L, pF29L-31L, pF29L-31L, pF29L-55V, or pF29L-55V to bombard the non-photosynthetic FBE strain N (Kuras et al., 1993; Kuras et al., 1995) which bears a deletion of the petA gene. Attempts to select clones growing on minimal medium under bright light proved unsuccessful: we recovered no phototrophic transformants. We then used plasmids pF29L-31L, pF29L-55V, pF29L-31L, pF29L-55V, and pF29L-55V to transform a wild-type (Wt) strain. Transformants were selected on TAP medium for the expression of the aadA cassette in the presence of 100 μg/ml spectinomycin. Transformants were then screened for the inactivation of cytochrome b₆f complexes based on their fluorescence induction kinetics after dark adaptation. Transformants displaying a lack of cytochrome b₆f activity were further purified by successive rounds of subcloning both on TAP-spectinomycin medium under dim light and on minimum medium (medium without acetate) under high light. When subcloning no longer generated colonies able to grow on minimal medium, transformants were considered as homoplasmic for the mutated genome. In parallel, the transformants were checked for their restriction fragment length polymorphism based on the presence of the new restriction sites brought along with the introduced mutations. At least three independent homoplasmic transformants were sequenced for each construct for further biochemical analysis. Their comparison with the wild-type was performed after having checked that there were no changes in the biogenesis of cytochrome b₆f subunits upon insertion of the aadA cassette at the EcoRV site in the intergenic petA-petD region in an otherwise wild-type context.

Protein Isolation and Separation—Biochemical analyses were carried out with cells grown in liquid TAP medium at a concentration of 5 × 10⁴ cells/ml. Soluble proteins were prepared from the supernatants of cells broken through a French pressure cell, as described in Kuras et al. (1995). The pellet was used to prepare purified thylakoid membrane as described by Chua and Bennoun (1975). Thylakoid membranes were twice shocked osmotically in order to remove soluble proteins trapped inside the membrane vesicles as described in Kuras et al. (1995). Before electrophoresis, proteins were solubilized in the presence of 2% SDS at 100°C for 50 s. Polypeptides were separated by SDS-polyacrylamide gel electrophoresis (12–18% polyacrylamide gradient) in the presence or absence of 8 M urea. Heme staining with TMBZ was performed as described by Thomas et al. (1976). Labeled polypeptides were detected by autoradiography of dried gels. Electrophoretograms were performed according to the method of Pierre (1983) using an enhanced chemiluminescence (ECL) method (Amersham International) according to the manufacturer’s protocol.

Pulse-Chase Labeling Experiments—Whole cells (2 × 10⁹ cells/ml) were concentrated 10 times and radiolabeled for 5 min with [³⁵S]cysteine, at a concentration of 10⁻⁴ M (5 μCi/ml) in the presence of 8 μg/ml cycloheximide as described by Depelpeaire et al. (1983). They were chased by addition of 10 volumes of TAP medium containing 50 μM acetate. 150 ml aliquots were removed after 0, 15, 30, 60, 120, and 180 min, mixed with 1 volume of chilled sodium acetate (50 mM), spun down at 4°C, resuspended in 50 ml 1,4-dithiothreitol/Na₂CO₃, and stored at −80°C.

Quantification of [³⁵S]Labeling—Dried gels, loaded with the radiola beled samples (at equal cell concentration), were analyzed with a PhosphorImager (Molecular Dynamics). Quantification of each form of labeled cytochrome f was achieved by measuring the difference between the signals arising from areas of equal value centered on the band of interest and in its immediate vicinity. Corrections were made for possible changes in [³⁵S] incorporation and in background labeling between each sample, conspicuous upon long chase periods, by normalization to an unrelated labeled band in the 15-kDa region whose stability remained unaltered throughout the chase.

Sucrose Gradient Experiments—Thylakoid membranes were solubilized in the presence of 25 mM hecamed (Vegatec, France) and loaded on a 15–45% sucrose gradient in the presence of both phosphatidylcholine and Hecamed according to the procedure of Pierre et al. (1995). After ultracentrifugation, fractions were collected and stored at −80°C until analysis.

The abbreviations used are: TMBZ, 3,3′,5,5′-tetramethylbenzidine; ECL, enhanced chemiluminescence.
RESULTS

To characterize the multistep process of cytochrome f biosynthesis, we constructed mutant strains of C. reinhardtii bearing specific alterations in the petA gene. Mutations were introduced as described under "Materials and Methods" to alter either heme attachment to the apoprotein (mutant F-52L-55V) or N-terminal processing of the precursor protein (mutant F-29L-31L). The same mutations were also introduced into a truncated version of the polypeptide sequence (mutants F-29L-31L-283ST and F-52L-55V-283ST). This mutation yields, in an otherwise wild-type context, a cytochrome b₆f-deficient strain which nevertheless accumulates a soluble, redox-active, and truncated cytochrome f in the thylakoid lumen (Kuras et al., 1995).

Heme Attachment Cytochrome f Mutants—Polypeptides from whole cells of the heme attachment transformants, F-52L-55V and F-52L-55V-283ST, were compared with those of the corresponding heme-containing strains, wild-type and F283ST. After urea-SDS-polyacrylamide gel electrophoresis, whole cell protein extracts were analyzed for their hemoprotein content by TMBZ staining (Fig. 1A) and for the presence of the major cytochrome b₆f subunits by immunoblotting (Fig. 1B). As expected, F-52L-55V and F-52L-55V-283ST transformants (lanes 3 and 4) failed to display heme-stained bands at the electrophoretic migration positions where full-length and truncated cytochrome f are observed in the wild-type and F283ST strains (lanes 1 and 2). This observation was consistent with a destruction of the covalent linkages for the heme cofactor. Nevertheless, the apoproteins could be detected with a specific polyclonal antibody although their abundance was greatly reduced. Their electrophoretic migration positions remained close to that of the full-length and truncated holoproteins (Fig. 1B). Therefore, we conclude that heme covalent binding is not a prerequisite for cytochrome f processing. Close examination of the gel in Fig. 1B shows that the apocytochromes migrated slightly ahead of their heme-containing counterparts (compare lanes 1/3 and lanes 2/4, respectively, on Fig. 1B). The F-52L-55V transformant (lane 3) displayed only trace amounts of apocytochrome f. Surprisingly, the F-52L-55V-283ST transformant (lane 4) accumulated much larger amounts of the truncated version of apocytochrome f, albeit to a lower extent than the heme-containing soluble cytochrome f in the F283ST strain (lane 2).

Both F-52L-55V and F-52L-55V-283ST transformants failed to accumulate significant amounts of the other cytochrome b₆f subunits. This is illustrated on Fig. 1B by the immunoblots reacted with subunit IV and Rieske protein antibodies. These subunits were present in trace amounts, comparable to that of the F283ST strain, and therefore are indicative of impaired assembly and/or stabilization of the cytochrome b₆f complex subunits (Kuras and Wollman, 1994).

Total protein extracts were further separated into soluble and membrane protein fractions by differential centrifugation. Cytochrome f from the wild-type strain is found exclusively in the membrane protein fraction (Fig. 1C). In spite of its trace amounts, the heme-less cytochrome f in its full-length version could also be detected exclusively in the membrane protein fraction (data not shown). In contrast, the truncated version of the heme-less cytochrome f was present in the soluble fraction (Fig. 1C) as was its heme-containing counterpart (Kuras et al., 1995). The corresponding membrane fractions retained trace amounts of truncated (apocytochrome f (experiment not shown), which were probably trapped inside the membrane vesicles created by the French Press treatment as we reported previously (Kuras et al. 1995). Thus, the truncated heme-less cytochrome f behaved as a soluble protein.

We then performed in vivo pulse-chase labeling experiments to determine whether the decreased amounts of heme-less cytochrome f in the mutant strains could be attributed to decreased synthesis or to higher turnover of the apoproteins. Fig. 2 shows the electrophoretic region of the autoradiogram where the labeled petA gene product migrates (indicated by arrows on Fig. 2, A, B, and C). During the chase period, the D2 band (see legend of Fig. 2) is often converted in a doublet, whose components D2.1 and D2.2 are produced in variable relative proportions (Delepelaire, 1984). They result from a slow post-translational phosphorylation of polypeptide D2 (Delepelaire and Wollman, 1985). This process, the regulation of which remains unclear, proved more active during the chases shown on Fig. 2C and Fig. 4, A and B, than during the chases shown on Fig. 2A and B.

Comparison of the pulse time points (0) shows that there was no decrease in the rate of synthesis of apocytochrome f in the transformants (compare the bands indicated by arrows in lanes 0, Fig. 2). In contrast, the lifetime of the newly synthesized forms of cytochrome f in the transformants was much shorter than in the wild-type. As we previously reported (Kuras and Wollman, 1994), holocytochrome f remained stable in the wild-type throughout the chase period (Fig. 2A). After 15 min of chase, a significant decrease in labeled apocytochrome f was already visible in F-52L-55V transformants (Fig. 2B) but not in F-52L-55V-283ST transformants (Fig. 2C). Quantification of the labeling by phosphorimaging (Fig. 2D) shows that the apocytochrome f is much more sensitive to proteolytic degradation when bound to the membrane (approximately τ₀/2 of 30 min) than when it is released as a soluble protein in the thylakoid lumen (approximately τ₀/2 of 120 min).

Cytochrome f Processing Mutants—We replaced the cleavage site (AQA) for the signal peptidase by an LQL sequence in the
of the precursor forms, it did not prevent some proteolytic cleavage of the N terminus extension.

The unprocessed form of truncated cytochrome f was heavily labeled during 5-min pulses of F-29L-31L-283ST transformant cells (Fig. 3B, arrow). Most of it turned over within 2 h of chase, with a half-time of about 45 min. We observed upon chase a possible conversion product of lower apparent molecular mass (asterisk, Fig. 3B), the low labeling and fuzzy aspect of which prevented quantification.

We then used specific antibodies to probe the cytochrome b₄ complex subunits which were expressed in the two processing mutants (Fig. 4A). A polyclonal antiserum raised against cytochrome f detected three bands in the F-29L-31L transformant (lane 3 on Fig. 4A) which migrated in this gel system at the same positions as the labeled bands f₁, f₃, and f₄ from Fig. 3A (experiment not shown). The three cytochrome f bands in F-29L-31L were also recognized by an antibody raised against a peptide from the C terminus of the protein (experiment not shown). Comparison of the electrophoretic migration positions shows that band f₃ has an apparent molecular mass about 1 kDa higher than that of mature cytochrome f, whereas band f₄ migrates slightly ahead of cytochrome f (compare lanes 1 and 3). Interestingly, the three cytochrome f bands, identified by antibody labeling, were TMBZ-stainable in purified thylakoid membranes (arrows, lane 3, Fig. 4B). The accumulation of the Rieske protein and subunit IV were similar in F-29L-31L transformants, to that in the wild-type (compare lanes 1 and 3 in Fig. 4A). A similar observation was made for cytochrome b₄ upon TMBZ staining of the membrane fraction (Fig. 4B).

Two cytochrome f bands where immunodetected in F-29L-31L-283ST transformants, the upper one corresponding to the
A

Fig. 4. **Cytochrome b,f complex subunits in cytochrome c processing mutants.** Immunoblots from urea-SDS-polyacrylamide gels loaded with total protein extracts from the WT (lane 1), F293ST (lane 2), F-29L-31L (lane 3), and F-29L-31L-283ST (lane 4) transformants reacted with antisera against cytochrome f (revealed with iodinated protein A), the Rieske protein and subunit IV (revealed by the ECL method) (A). Same gel system as for A was loaded with purified membranes and stained with TMBZ; arrows point to the three heme-binding forms of cytochrome f in F-29L-31L transformants; h1 and h2 correspond to mitochondrial cytochromes contaminating thylakoid preparations (Attea et al., 1992) (B).

B

one observed in 5-min pulse-labeling (lane 4 in Fig. 4A). A further analysis of the total protein extract from the F-29L-31L-283ST strain showed that the precursor form was enriched in the membrane fraction whereas the form of lower molecular weight was released in the soluble protein fraction (experiment not shown). TMBZ staining of the thylakoid membranes detected trace amounts of truncated cytochrome f (not visible on Fig. 4B). The content of other cytochrome b,f subunits in the F-29L-31L-283ST strain (lane 4, Fig. 4A) was decreased to about the same extent as in the F293ST strain which fails to assemble the protein complex (lane 2, Fig. 4A). Cytochrome b6 remained below detection upon TMBZ staining (lane 4, Fig. 4B) a situation similar to the one we previously reported for F283ST transformants (Kuras et al., 1995).

Since all the major subunits of the cytochrome b,f complex accumulated in the F-29L-31L mutant, we investigated the state of assembly of the three heme-stainable forms of cytochrome f. Detergent-solubilized preparations of thylakoid membranes from the wild-type and F-29L-31L strains were loaded on a continuous 10–35% sucrose gradient (Pierre et al., 1995). After centrifugation, the distribution profiles of cytochrome f were analyzed using specific antibodies. Cytochrome f, cytochrome b6, and subunit IV comigrated among the gradient fractions from the wild-type (Fig. 5A) and mutant (Fig. 5B). A single peak, centered around a sucrose density of 22%, was observed with the wild-type. It corresponded to the migration of the mature cytochrome f dimers which are preserved by this method of purification (Pierre et al., 1995). The distribution of cytochrome b6 complex subunits in the transformant was bimodal, with a major peak similar to that in the wild-type (22% sucrose) and a minor peak at higher density (28%). The three forms of cytochrome f (f1, f3, f4) were found in association with cytochrome b6 and subunit IV. The precursor form f1 is particularly visible in the fractions of higher sucrose density. We conclude that the three forms of cytochrome f, the precursor form and the two forms of lower molecular weight, were competent for assembly in cytochrome b6 complexes.

Comparison of Rates of Synthesis of Cytochrome f in Wild-type and Transformants—We previously reported that the rate of synthesis of cytochrome f, measured by 5-min pulse-labeling, varied widely between strains which differ in the final localization of cytochrome f, depending on its assembly in cytochrome b,f complexes (Kuras and Wolman, 1994) or whether it becomes soluble in the thylakoid lumen (Kuras et al., 1995). We therefore compared its rates of synthesis in the various transformants. Fig. 6, A and B, shows comparative migration patterns of the various forms of cytochrome f upon electrophoresis on acrylamide gels containing (Fig. 6A) or lacking (Fig. 6B) 8 M urea. The former provides a better resolution of the changes in molecular weights due to carboxyl-terminal truncation of cytochrome f. Heme-less cytochrome f of full-length (lane 2) migrates slightly ahead of genuine cytochrome f (lane 1). The corresponding truncated forms (lanes 3 and 4) display a similar increase in electrophoretic mobility due to a polypeptide sequence shorter by 35 residues. The unprocessed but truncated cytochrome f (lane 6) migrates at a position close to that of genuine cytochrome f which is consistent with the absence of the last 35 amino acids from the carboxyl terminus together with the presence of the 31 residues from the signal sequence. However, this gel system does not resolve the precursor form of cytochrome f which comigrates with polypeptide P6 (upper band on Fig. 6A). In the absence of urea, the precursor form is resolved as a doublet of two closely migrating bands of lower mobility than mature cytochrome f (compare lanes 1 and 6 of Fig. 6B). By analogy with the slightly higher electrophoretic mobility of apocytochrome f (lane 2) versus holocytochrome f (lane 1), we tentatively assign the lower band of the doublet, the short-lived form f2, to pre-apocytochrome f whereas the upper one, the heme-stainable form f1, corresponds to pre-holocytochrome f. Quantification of the labeling...
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**Fig. 6.** Comparative rates of synthesis of cytochrome f forms. Upon 5 min of pulse-labeling of chloroplast-encoded polypeptides from the WT (lane 1), F-52L-35V (lane 2), F-283ST (lane 3), F-52L-35V-283ST (lane 4), F-29L-31L (lane 5), and F-29L-31L-283ST (lane 6) strains. Labeled polypeptides were resolved upon polyacrylamide gel electrophoresis in the presence (A) or absence (B) of urea. Cytochrome f synthesis in each strain was quantified by phosphorimaging, relative to that in the wild-type (C).

![Graph](http://www.jbc.org/)

| Strains  | 1   | 2   | 3   | 4   | 5   | 6   |
|----------|-----|-----|-----|-----|-----|-----|
| Rate of synthesis (%) | 100 | 252 | 289 | 325 | 96  | 324 |

DISCUSSION

**Heme Binding Is Not Required for Processing of the Leader Peptide.—** In bacteria, mitochondria, and chloroplasts, heme attachment to c-type cytochromes occurs upon transfer of most, or all, of the apoprotein through a biological membrane. The enzymes involved in heme attachment are most likely located next to the side of the membrane opposite to the site where translocation of the apoprotein is initiated (for a review, see Howe and Merchant (1994)).

The interplay between heme attachment and membrane translocation has been addressed for both the soluble and membrane-bound c-type cytochromes in the mitochondria. In each case, heme attachment seems to be required for proper localization of the final protein. It has been proposed that soluble cytochrome c is trapped irreversibly in the intermembrane space by covalent ligation of the heme (Dumont et al., 1991) whereas heme attachment is required for the proper processing of membrane-bound cytochrome c (Nicholson et al., 1989).

In the case of chloroplast cytochrome f, a plastome mutant from Oenothera described as defective in heme binding to (pre)apocytochrome f showed a decreased efficiency in processing of the precursor form (Johnson and Sears, 1990). In contrast, previous studies based either on nuclear mutants from C. reinhardtii probably defective in c-heme attachment in the chloroplast (Howe and Merchant, 1992) or upon gabaculin treatment aimed at decreasing heme availability (Anderson and Gray, 1991; Howe et al., 1995) led to the conclusion that cytochrome f processing occurs in the absence of heme binding. The present study, employing a reverse molecular genetic approach, supports the latter view. We destroyed the heme binding sites by replacement of the two cysteinyI ligands by a leucine and a valine. We observed that the altered cytochrome f, although synthesized at higher rates than in the wild-type (see below for further discussion), was highly unstable and therefore prevented significant accumulation of the other sub-units of cytochrome b_{6}f complexes. However, the mutations still allowed conversion of pre-apocytochrome f to its processed form as well as its complete thylakoid translocation as demonstrated by the release of a soluble protein in a strain lacking the C-terminal membrane anchor of cytochrome f. Thus, the biogenesis of membrane-bound cytochrome f resembles that of soluble cytochrome c_{6}, the other c-type cytochrome in the chloroplast (Howe and Merchant, 1994); translocation and processing of precursor forms of the two proteins are independent of heme binding.

**Rapid N-Terminal Processing Is Not a Prerequisite for Heme Binding or for Cytochrome f Assembly in Cytochrome b_{6}f Complexes.—** In the present study, we observed that replacement of the AQA consensus site for the processing peptidase by an LQL sequence, delayed, but did not abolish, processing of the precursor form of cytochrome f. The radiolabeled precursor appeared as a doublet of pre-holocytochrome f and a band of slightly higher mobility which we tentatively assigned to pre-apocytochrome f. The precursors were then converted into two forms of lower molecular weights which display electrophoretic migration positions on both sides of mature cytochrome f. Comparison of a number of sequences of lumenal-targetted proteins supports a stringent requirement in short side chain residues at the −3 and −1 positions for the proteolytic processing of signal peptides (von Hejne et al., 1989; Robinson and Klösgen, 1994). In particular, alanine is consistently present at all −1 positions. Therefore, the two processed forms may result from improper cleavage at upstream and downstream alanine residues with respect to the cleavage site in the wild-type. Alanines are found in a suitable context at the −11 position, a GLA site, and at the +5 position, a VFA site, in the sequence of pre-apocytochrome f (Büschen et al., 1991).

The crystal structure of cytochrome f (Martinez et al., 1994) shows that one axial ligand is provided by the α-amino group of the N terminus released upon processing of the precursor protein. It was then tempting to suggest that conversion of pre-apocytochrome f to the mature form would occur through an orderly process opposite the one advocated for mitochondrial cytochrome c (Nicholson et al., 1989); the protein conformation suitable for heme attachment would require processing of its precursor form. In contrast to this view, the delayed processing in F-29L-31L transfectants allowed us to observe heme binding to both the precursor and processed forms of cytochrome f. Moreover, these three heme-stainable forms of cytochrome f were detected within assembled cytochrome b_{6}f complexes. Therefore, we conclude that rapid cytochrome f processing is not required for heme attachment or for the assembly of the protein into cytochrome b_{6}f complexes. This observation does not necessarily imply that heme binding occurs prior to protein processing in the wild-type strain. Delayed kinetics of processing may have elicited a branched pathway for direct conversion of pre-apo- to pre-holocytochrome f. It nevertheless implies that, were the maturation processes to occur in two subsequent steps in the wild-type, they would be primarily determined by a kinetic competition rather than by mechanistic constraints.

Since the delayed processing in the F-29L-31L transfectants yielded assembled but inactive cytochrome b_{6}f complexes, we presently assume that an improper liganding environment has
prevented the attached heme from adopting a suitable orientation and that its redox potential may be modified. Further biophysical studies will be required to elucidate this point.

Accumulation of Misfolded Cytochrome f Is Prevented by a Membrane-bound Proteolytic System—We observed that apocytochrome f was only unstable, whereas its truncated counterpart, soluble in the lumen, was largely protected against degradation. This is not merely due to the absence of a proteolytic site within the C-terminal anchor since the truncated version of the processing mutant was also highly sensitive to degradation. In the latter case, we observed that the precursor form was a short-lived species by pulse-chase labeling experiments, but we failed to detect by autoradiography the form of lower molecular weight that we identified on immunoblots. This is most easily explained by a conversion process of low efficiency from a short-lived species, bound to the membrane by the signal peptide, to a long-lived species, soluble in the lumen due to proteolysis of the signal sequence. Thus, we conclude from the comparison of the lifetimes of the various altered forms of cytochrome f in the transformants that either membrane assembly with cytochrome $b_6f$ subunits or release in the lumen provides protection against proteolytic degradation.

If the degradation process was initiated from the stromal face of the thylakoids, we would expect a release of the large amino-terminal domain of the protein in the lumen upon proteolytic attack of the carboxyl terminus anchor. This mechanism would mimic the situation we created by truncating cytochrome f at its carboxyl terminus. Since we observed that the free soluble amino-terminal domain is then rather stable (this work and Kuras et al. 1995), we conclude that the proteolytic system responsible for recognition and degradation of misfolded cytochrome $f$ is intimately associated with the thylakoid membranes and has access to the protein via the luminal side of the membrane.

The Presence of the Carboxyl-terminal Membrane Anchor Regulates the Rate of Synthesis of Cytochrome $f$—The rate of synthesis of cytochrome $f$, measured by 5-min pulse-labeling in vivo, is a tightly controlled process. We have shown previously that it drops in the absence of other subunits of the cytochrome $b_6f$ complex (Kuras and Wollman, 1994). On the other hand, we observed in this study that it increases in the absence of heme binding. A conspicuous feature of the carboxyl-terminal truncation of cytochrome f is the 3-fold increase in its rate of synthesis as compared to that of full-length cytochrome f. This stimulation appears to be independent of the lifetime of the resulting protein product since the truncated, redox-active cytochrome is stable for hours whereas forms lacking the heme-binding cyanines or the processing site are shorter-lived species, with lifetimes of approximately 120 min and 45 min, respectively. The apparent stimulation of synthesis could be attributed to the lower accessibility of a membrane-bound protease, because of the rapid release of the truncated cytochromes in the thylakoid lumen. However, our observation that the unprocessed but truncated form of cytochrome f, which is membrane-bound by the signal sequence, displays a similar increase in synthesis, argues against this hypothesis. Our data instead support a negative control of cytochrome f synthesis by the carboxyl-terminal domain of the protein. The regulatory site would be shielded upon assembly in cytochrome $b_6f$ complexes. This hypothesis would be consistent with the drop in cytochrome $f$ synthesis in the absence of subunit IV or cytochrome $b_6$ owing to the presence of long-lived cytochrome f, albeit at 50% of wild-type levels, with a free carboxyl terminus domain available for a negative control (Kuras and Wollman, 1994). In contrast, the absence of this domain in the truncated versions of cytochrome f, as well as its low concentration in the heme-binding mutation F-52L-55V due to the short lifetime of apocytochrome f, would produce a release of the negative control. Whether the carboxyl terminus domain contains a specific site for a protease active on nascent chains of cytochrome f or whether it controls the rate at which translation of the petA message is completed or reinitiated requires further investigations.

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