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The 4-kDa Nuclear-encoded PetM Polypeptide of the Chloroplast Cytochrome b_6f Complex

NUCLEIC ACID AND PROTEIN SEQUENCES, TARGETING SIGNALS, TRANSMEMBRANE TOPOLOGY*

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The 4-kDa subunit of cytochrome b_6f complex encoded by the nuclear *PetM* gene in *Chlamydomonas reinhardtii* has been characterized. 38 of the 39 residues of the mature protein have been established by Edman degradation, a cDNA clone encoding the complete precursor has been isolated and sequenced, and a 0.6-kb transcript detected. The deduced amino acid sequence of the precursor includes an N-terminal transit peptide of 60 amino acids with stromal targeting features. Examination of the sequence suggests that PetM spans the membrane as a single transmembrane α-helix, which is supported by its non-extractability following dissociating treatments. When PetM and PetG, another small subunit of the b_6f complex, are folded into α-helices, an array of identical residues becomes apparent. Proteolysis data, charge distribution, and homology with PetG are consistent with a lumenal localization of the N terminus of PetM.

The cytochrome b_6f complex catalyzes electron transfer from plastoquinol to an acceptor protein (plastocyanin or a soluble cytochrome) in the photosynthetic membrane of plants, algae, and some bacteria. A homologous complex, cytochrome bc_1, plays a comparable role in mitochondria and in several prokaryotes. The cytochrome b_6f complex of *Chlamydomonas reinhardtii* (1–3) is similar to that of higher plants (4). It is comprised of four high molecular mass subunits: cytochrome *f* (*petA* gene product), cytochrome b_6 (*petB* gene product), subunit IV (*petD* gene product) (homologous to the C-terminal region of mitochondrial cytochrome *b*), all three of them chloroplast-encoded, and the Rieske iron-sulfur protein (*PetC* gene product), which is nuclear-encoded. Three hydrophobic and very small (~4 kDa) proteins are also present: two of them, PetG (*petG* gene product) and PetL (*petL* gene product), are chloroplast-encoded and one, PetM (*PetM* gene product), is nuclear-encoded. PetG has been characterized as a b_6f subunit in maize (5) and identified in *C. reinhardtii* b_6f complex (6, 7). PetL has been characterized by deletion of the chloroplast *petL* gene and immunoblot analysis of purified b_6f complex in *C. reinhardtii* (8). Gene *petL* is also present in higher plant chloroplast genomes.

Concerning PetM, we have previously identified by protein sequencing and immunodetection a 4-kDa polypeptide that is present in purified b_6f complexes and absent in b_6f-deficient mutants from *C. reinhardtii* (7); we provisionally designated this novel b_6f subunit PetX, product of the nuclear *petX* gene (7, 9). To conform to the alphabetical order recommended for gene nomenclature, we shall from now on refer to this protein as PetM (b_6f complex subunit 7). PetM has been independently identified in *C. reinhardtii* and in spinach b_6f by Schmidt and Malkin (6). PetM is translated as a precursor in the cytoplasm and imported into the chloroplast. In the present work, we have determined 38 of the 39 residues of the mature protein by Edman degradation, isolated, and sequenced a cDNA clone encoding the complete precursor of PetM, whose transit peptide has features of a stromal-targeting peptide, and studied PetM transmembrane topology by dissociating treatments and trypsinization.

EXPERIMENTAL PROCEDURES

Protein Sequencing—b_6f complex was purified by solubilization of thylakoid membranes with 6-*O*-(*N*-heptylcarbamoyl)-methyl-α-D-glycopyranoside, centrifugation on sucrose density gradient, and hydroxylapatite chromatography (3). After urea/SDS-PAGE, purified b_6f complex was electrotransferred onto Prot Blott™ membranes (Applied Biosystems) in a semidry blotting system at 0.8 mA/cm^2 for 30 min (10). Sequencing of the 4 kDa band by Edman degradation was performed by J. d'Alayer (Laboratoire de Microséquençage des Protéines, Institut Pasteur, Paris). This band contains PetG, PetL, and PetM, but under our experimental conditions the only unblocked N terminus is that of PetM (7–9, 11).

Screening of cDNA Library, DNA Sequencing, and RNA Analysis—A *C. reinhardtii* cDNA library cloned into λ-GT10 (12), kindly provided by M. Goldschmidt-Clermont (University of Geneva), was replicated in *Escherichia coli* C600. Screening by plaque hybridization and isolation of positive clones were performed using oligonucleotide primers based on the amino acid sequence of the N terminus of PetM (Fig. 2) and [^{32}P]ATP-phosphorylated by T4 polynucleotide kinase. The *Eco*RI insert was isolated from one of the phage isolates, subcloned into Bluescript KS$^-$, and sequenced by the dideoxy chain termination method. Total RNA was analyzed as described in Ref. 13 using as a probe the *Eco*RI insert encoding PetM.

Preparation of Antipeptide Antisera—An antiserum was raised against a synthetic peptide corresponding to the C terminus of PetM (Fig. 1), coupled to ovalbumin by an additional tyrosine at its N terminus, as described in Ref. 14. We have described elsewhere the antisera against PSII subunit OEE3 (15), the N terminus of PetM (7), and subunit IV (3).

Protein Electrophoresis, Transfer, and Immunoblotting—Urea/SDS-PAGE and electrotransfer were performed as described previously (10). Immunodetection was carried out by labeling either with ^{125}I-protein A using anti-PetM antisera at a dilution of 1/50 or with the enhanced chemiluminescence method (Amersham Corp.) using anti-OEE3 and anti-subunit IV antisera at dilutions of 1/20,000 and 1/10,000, respectively. Procedures were as described in Ref. 14.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) U36401.

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GEAEFIAGTALTMVGMTLVGLAIGFVLLRVESLVEEGK

FIG. 1. **N-terminal sequence of PetM obtained by protein sequencing.** *Bold italics* indicate the sequences of the synthetic peptides used to raise antibodies. Sequences used to design oligonucleotide probes are *underlined*.

3'-CCGAAGCAC/GGACGACGCGCA-5'(GFVLLRV)

3'-GACCAC/GCTCCTCCCGTTC-5' (LVEEGK)

FIG. 2. **Oligonucleotides used for cloning and corresponding amino acid sequences.**

```
                                                        50
GGCCAACAAAATGGCCATGTCCATCGCTGCTCGCTCGGCTTGCTGCGGCGTCGCTGCGCC
           M  A  M  S  I  A  A  R  S  A  C  C  G  V  A  A  P
                                                   100
CCGCAGCTCGACCGTCCGCGTCGCTGCTGCCCGGCCAGCTGTCCGCCCGTCCCTCAGGAC
 R  S  S  T  V  R  V  A  A  A  R  P  A  V  R  P  S  L  R  T
                                         150
CGCCGGCCAGAAGGCTGCCCCCAGCCGCGGTGTGGCCACCAAGGCCGTGAACGAGCTGGC
 A  G  Q  K  A  A  P  S  R  G  V  A  T  K  A  V  N  E  L  A
                               200
CATGATTGCCGGCGAGGCTGAGTTCATTGCTGGCACCGCCCTGACCATGGTCGGCATGAC
 M  I  A ↓G  E  A  E  F  I  A  G  T  A  L  T  M  V  G  M  T
            G  E  A  E  F  I  A  G  T  A  L  T  M  V  G  M  T
         250                                              300
CCTGGTGGGTCTGGCCATTGGCTTCGTGCTGCTGCGCGTGGAGTCTCTGGTTGAGGAGGG
 L  V  G  L  A  I  G  F  V  L  L  R  V  E  S  L  V  E  E  G
 L  V  G  L  A  I  G  F  V  L  L  R  V  E  S  L  V  E  E  G
                                                 350
CAAGATCTAAGCTCGCTCAGTACTCGTGTCCTTGTGTGGGTCTGCATTTGAGAAGAGAGT
 K  I ---
 K
                                                   400
GGGGTTCGGGGACTGTTCGCCACCGGCGTTGAGTGCTGACCACGCTGGTCACGCCGTCGG
                                         450
ATTGGCCGGGGGCACGCAGGGCGTGATTTTCGGGGCACGTCGCCTCCCAGGGGGGAAGCA
                               500
GCGACCTGCCGATGGCGCGGTTTTGCCACGATTCAGCGCCGCTTGTAACGCCGCGCCTGC
         550
CAAAAAAAAAAAAAA
```

FIG. 3. **Nucleotide and amino acid sequence of PetM (EMBL accession number X92488).** The deduced amino acid sequence is in **bold type**, the amino acid sequence determined by Edman degradation is in *italics*. The *arrow* indicates the processing site. The hydrophobic region likely to correspond to a transmembrane α-helix is *underlined*.

Polypeptide Extraction from Thylakoid Membranes—Polypeptide extraction from thylakoid membranes was performed as described in Ref. 14. Thylakoid membranes were incubated with a dissociating agent (6.8 M urea, 2 M KSCN, or 1.5 M NaI in Tricine[1] buffer, pH 8.0, or 20 M CAPS, pH 12.0) for 10 min at room temperature, frozen/thawed, and centrifuged. In order to improve the immunodetection of PetM, the pigments and lipids of the pellets were extracted by incubating 15 μl of the resuspended pellet in 100 μl of ice-cold methanol for 10 min, followed by addition of 900 μl of ice-cold ether, 10-min incubation on ice, and centrifugation for 10 min at 12,500 $\times$ *g*. The resulting pellet was resuspended in 15 μl of 100 mM dithiothreitol, 100 mM Na_2CO_3, 2.5% SDS solution, and analyzed by urea/SDS-PAGE along with the supernatant of the first extraction.

Trypsinization of Thylakoid Membranes—Thylakoid membranes (16) were washed twice with 10 mM Tris, pH 7.8, without protease inhibitors. Various concentrations (from 0 to 25 μg/ml) of trypsin (L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated, type XIII from bovine pancreas, Sigma) were added to thylakoid membranes at a final chlorophyll concentration of 1 mg/ml. Half of each sample was sonicated for 20 s on ice. Trypsin treatments were performed at room temperature for 1 h and the reaction arrested by methanol/ether extraction. The resulting pellet was resuspended in 100 mM dithiothreitol, 100 mM Na_2CO_3, 2.5% SDS, and proteases inhibitors (200 μM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 5 mM ϵ-aminocaproic acid) and analyzed by urea/SDS-PAGE and immunoblotting. The efficiency of proteolysis blockade is demonstrated by the differential cleavage of lumenal proteins depending on whether thylakoid membrane vesicles are disrupted by sonication or not.

Hydrophobicity Analysis—The presence of putative transmembrane hydrophobic α-helices was assessed using the GES scale (17) and a 17-residue window (18). Sequences are from Refs. 9, 19, and 20.[1]

RESULTS AND DISCUSSION

N-terminal Amino Acid Sequence of PetM—Sequencing of PetM by Edman degradation yielded 38 residues, which corresponds almost to the entire mature protein (Fig. 1). Our previous data were limited to 28 residues (7). The additional information allowed us to design better oligonucleotide probes and to raise an antipeptide antiserum recognizing PetM C terminus.

Nucleic Acid Sequence and Messenger RNA—Based on this amino acid sequence, two oligonucleotide primers (Fig. 2) were synthesized and used to screen a *C. reinhardtii* cDNA library cloned into λ-gt10 (12). The *Eco*RI insert isolated from one of the phage isolates was subcloned into Bluescript KS^- and sequenced. The insert was 556 bp long with a putative polyadenylation signal located 13 nucleotides upstream from the poly(A) tail (Fig. 3). The transcript of the *PetM* gene is 0.6 kb long (Fig. 4), which corresponds to the size of the insert.

The Transit Peptide: Similarity to a Stromal-targeting Peptide—There are two in-frame ATG codons at the beginning of the *PetM* coding sequence. The deduced amino acid sequence begins with MetAlaMet as in the *C. reinhardtii* Rieske protein (21). *C. reinhardtii* initiation regions have a consensus sequence with AUG generally preceded by an A-rich tetranucleotide similar to that of higher plants AACAAUGGC (see Ref. 21 and references therein). Therefore, the first methionine codon

[1] The abbreviations used are: Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; OEE, oxygen evolution enhancer; PAGE, polyacrylamide gel electrophoresis.

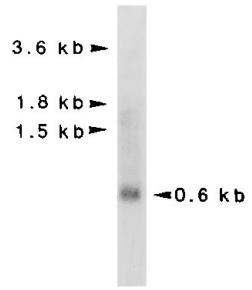

FIG. 4. **Northern blot of total RNA from *C. reinhardtii* wild-type cells probed with PetM cDNA.** Ribosomal RNA was used for molecular weight determination.

(CAAAATGGC) seems more likely to be the initiation codon than the second (GGCCATGTC). Starting at the first ATG, the precursor protein is 99 residue long with a molecular mass of 10.0 kDa. The site of processing is indicated by the N-terminal sequence of the mature protein (6, 7, 9). Starting at the first ATG, the transit peptide is 60 residues long with a molecular mass of 6.0 kDa, which is larger than the mature protein. It comprises (i) a short uncharged N-terminal region, (ii) a central region rich in residues with basic (Arg, Lys) or small (Ala, Ser) side chains, (iii) an alanine residue preceding the processing site (Fig. 3). These features are typical of transit peptides directing transfer through the chloroplast envelope (22, 23). However, at variance with the transit peptides of most of the proteins transferred through the thylakoid membrane (22, 24), that of PetM contains no hydrophobic segment in its C-terminal region (Fig. 5) and has only features of a stromal-targeting sequence.

The Mature Protein: a 4-kDa Hydrophobic Protein—The mature protein is 39 residues long with a molecular mass of 4.0 kDa, matching its apparent molecular mass in urea/SDS-PAGE of ~4 kDa (7) or 3.8 kDa (6). The sequence of the mature protein established by Edman degradation is identical to that deduced from the cDNA sequence, except that it lacks the last residue (Fig. 3), which probably originates from the low yield often observed for the last amino acid in protein sequencing. The mature protein features a 24-residue hydrophobic segment likely to form a transmembrane α-helix (Fig. 5). The average hydrophobicity of the most hydrophobic 17-amino acid residue stretch of PetM (2.3 kcal/residue on the GES scale) is among the highest among putative transmembrane α-helices of b_6f subunits (b_6, 1.9 (average of four α-helices); IV, 2.2 (average of three α-helices); and *f*, 2.3; PetG, 2.2; PetL, 2.2 (1 α-helix)). A systematic examination of hydrophobic segments in the sequences of integral membrane proteins has revealed no segments with such a high hydrophobicity that would not be transmembrane (18). The lateral amphipathy of the putative α-helix is low.

Transmembrane Topology: a Single α-Helix with Short Extramembrane Segments—The mode of association of PetM with the thylakoid membrane was probed using various dissociating treatments. Cytochrome *f*, cytochrome b_6, subunit IV, PetG, and PetL cannot be extracted by these treatments (14),[1] indicating that they are intrinsic membrane proteins, whereas the Rieske protein is extrinsic (8, 14). Thylakoid membranes were incubated for 10 min in the dissociating solutions, subjected to two freeze/thaw cycles, centrifuged, and the pellet and supernatant analyzed by urea/SDS-PAGE and immunoblotting. As shown in Fig. 6, PetM is not extracted by dissociating treatments (6.8 M urea; 2 M KSCN; 1.5 M NaI; 20 mM CAPS, pH 12.0), as is the case for transmembrane polypeptides (*e.g.* subunit IV (*IV*) in Fig. 6) and in contrast to peripheral polypeptides (*e.g.* polypeptide OEE3 (*OEE3*) in Fig. 6).

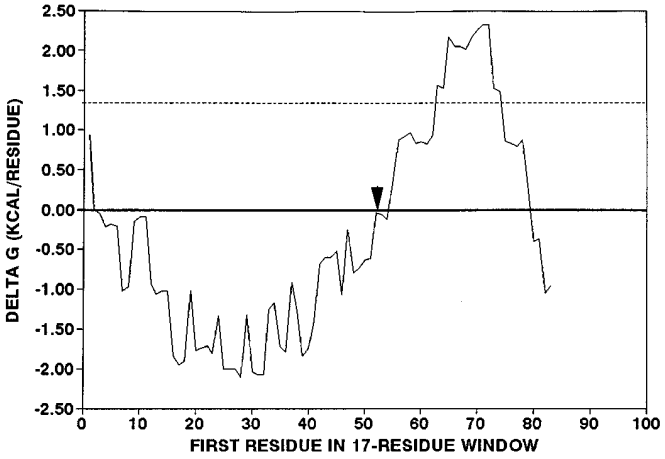

FIG. 5. **Hydrophobicity profile of PetM precursor.** The free energy of transfer from lipids to water calculated using the GES scale has been averaged over a 17-residue window. ▼, processing site. In integral membrane proteins most sequence segments with average hydrophobicity > 1.3 kcal/residue (*dotted line*) correspond to transmembrane helices (18).

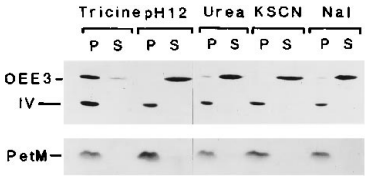

FIG. 6. **Extraction of thylakoid proteins by various dissociating treatments.** Thylakoid membranes were treated either with 20 mM Tricine, pH 8.0, or with 6.8 M urea, 2 M KSCN or 1.5 M NaI in Tricine, pH 8.0, or with 20 mM CAPS, pH 12.0. *S*, supernatant, *P*, pellet. The immunoblot was labeled with antisera directed against the extrinsic protein OEE3 (*OEE3*), the intrinsic subunit IV (*IV*), or the N terminus of PetM (*PetM*).

Purified preparations of b_6f complex contain one molecule of chlorophyll *a* per cytochrome *f* (3, 4, 11, 25). A spectral shift associated with functioning of the b_6f suggests that it is located in the vicinity of the Q_0 site (26). Our attempts to detect chlorophyll *a* binding to PetM using low temperature SDS-PAGE (27) have been unsuccessful. Subunits with a single transmembrane α-helix and short extramembrane segments are numerous in the thylakoid membrane and inner mitochondrial membrane complexes (18, 28). Most of them have no known cofactors. Deletion of their coding gene usually destabilizes the complex to which they belong, as is the case for PetG (29) and, to a lesser extent, for PetL (8). Directed deletion of nuclear genes is difficult to achieve in *C. reinhardtii* because of the low efficiency of homologous recombination. It is not yet known whether PetM is also present in prokaryotic photosynthetic organisms, in which directed mutagenesis is more efficient.

N Terminus: Indications for a Localization on the Lumenal Side of the Membrane—The "positive-inside" rule applies to thylakoid membrane proteins, regions enriched in basic residues on average facing the stroma (30). The presence of basic residues in the C terminus of PetM suggests its stromal localization. In order to test this prediction, thylakoid membranes were trypsinized for 1 h at room temperature, with and without sonication. Trypsin has two potential cleavage sites in the C terminus of PetM (Arg_{29} and Lys_{38}). Blots were probed with antisera raised against peptides corresponding to the N terminus and C terminus of PetM (Fig. 7*A*). While the specificity of the antisera is not complete (particularly as regards the C terminus antiserum, which is very weak), immunostaining of

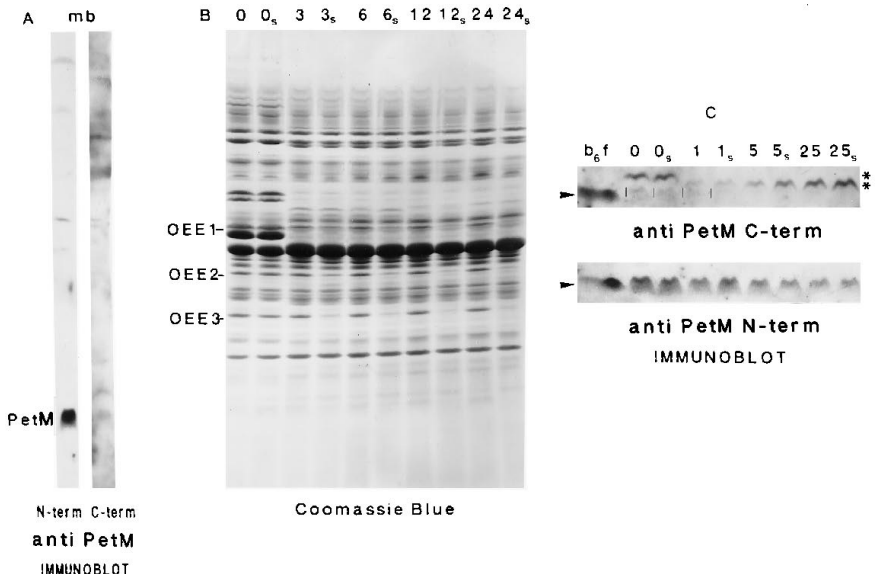

FIG. 7. **Proteolysis of thylakoid membrane proteins by trypsin.** Analysis by urea/SDS-PAGE. *A*, PetM immunodetection on methanol/ether extracted membranes using antisera raised against the N terminus (*N-term*) and the C terminus (*C-term*); *mb*, membranes. *B*, polypeptide pattern after incubation with increasing trypsin concentrations (0, 3, 6, 12, and 24 μg/ml); *s*, vesicles were sonicated in the presence of trypsin; gel was stained with Coomassie Brilliant Blue; note the effect of sonication on the degradation of the lumenal protein OEE3. *C*, PetM immunodetection after incubation with trypsin. b_6f, purified b_6f complex; the polypeptide band noted (▶) in the purified b_6f indicates PetM; the two cross-reacting bands noted by the *asterisks* are a thylakoid membrane polypeptide detected in the absence of trypsin and a degradation product detected in its presence.

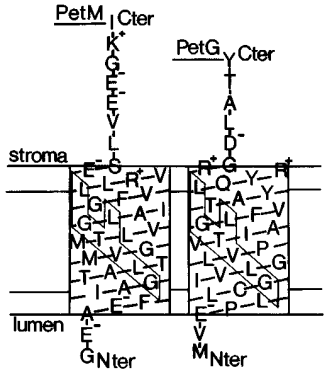

FIG. 8. **Putative transmembrane arrangement of PetM and PetG.** PetG sequence from Ref. 29. Identical residues are *boxed*.

the blots in the 4-kDa region is specific for PetM, since it is not observed when membranes from the b_6f-less *C. reinhardtii* mutant FUD4 are used (Ref. 7 and data not shown). In contrast to lumenal proteins, whose trypsinization increases when thylakoid vesicles are disrupted by sonication (*e.g. OEE1, OEE2*, and *OEE3* in Fig. 7*B*), PetM was cleaved by trypsin to a similar extent in both intact and sonicated vesicles (Fig. 7*C*). As revealed by antisera against PetM N and C termini, intact and proteolyzed PetM are not resolved by urea/SDS-PAGE (Fig. 7*C*) (PetM, PetG, and PetL also comigrate in this gel system; Refs. 7–9 and 11). Decreased immunolabeling with the antiserum directed against PetM C terminus was expected from proteolysis from the stromal side; it did decrease to a similar extent in sonicated and intact vesicles with increasing trypsin concentrations from 0 to 5 μg/ml (Fig. 7*C*). Labeling with the anti-N terminus was still strong after exposure to trypsin at 25 μg/ml, as expected from the absence of a trypsin cleavage site in PetM N terminus. However, the labeling with anti-PetM-N terminus decreases with increasing trypsin concentrations. This may reflect a change of electrophoretic migration following cleavage of PetM. Altogether, these data suggest that the C terminus of PetM is exposed to the stroma.

PetG has been shown to also feature a single transmembrane α-helix whose N terminus is lumenal (5). Residue identities between PetM and PetG are limited (29% in the putative transmembrane region). However, a zone of identity becomes apparent when PetM and PetG are modeled to form α-helices (Fig. 8). This similarity (which is not observed with PetL) suggests either a common ancestry or convergent evolution. If not coincidental, it may indicate that PetG and PetM share similar functions and, presumably, span the membrane with the same orientation, *i.e.* a lumenal N terminus, in keeping with the distribution of basic residues in their sequences as well as with proteolysis data (Fig. 8). Determination of PetM sequences from other organisms will be necessary in order to test the generality of this observation. Gene duplication has been proposed previously to account for similarities between the nuclear-encoded subunit II and the chloroplast-encoded subunit I from *C. reinhardtii* CF_0, both of which are homologous to F_0 subunit b from *E. coli* (31).

PetG is chloroplast-encoded and has no transit peptide (5). We show here that PetM has a transit peptide with only the features of a stromal-targeting peptide. Nonetheless, a lumenal localization of the N termini of both proteins implies that these can be translocated across the thylakoid membrane. In bacteria, translocation of N termini across the inner membrane is most efficient when they are short and contain few positively charged residues; translocation in these cases is Sec-independent and depends on the membrane potential (32). The N termini of PetG and PetM are both very short and very poor in positive charges. Analogy with bacteria would suggest that their translocation is Sec-independent and dependent on the membrane potential. Studies of this process should lead to further insights into the mechanisms of protein translocation across thylakoid membranes.

Conclusion—Three b_6f subunits belonging to the class of proteins forming a single transmembrane α-helix with short extramembrane segments have now been characterized. Two are chloroplast-encoded, PetG (5) and PetL (8). One is nuclear-encoded, PetM (6, 7, 9); the present work establishes the cDNA sequence of *PetM*, as well as the amino acid sequence, the targeting signals and the transmembrane topology of its product.

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