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Molecular Genetic Identification of a Pathway for Heme Binding to Cytochrome b_6*

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Heme binding to cytochrome b_6 is resistant, in part, to denaturing conditions that typically destroy the noncovalent interactions between the b hemes and their apoproteins, suggesting that one of two b hemes of holocytochrome b_6 is tightly bound to the polypeptide. We exploited this property to define a pathway for the conversion of apo- to holocytochrome b_6, and to identify mutants that are blocked at one step of this pathway. *Chlamydomonas reinhardtii* strains carrying substitutions in either one of the four histidines that coordinate the b_h or b_l hemes to the apoprotein were created. These mutations resulted in the appearance of distinct immunoreactive species of cytochrome b_6, which allowed us to specifically identify cytochrome b_6 with altered b_h or b_l ligation. In gabaculine-treated (*i.e.* heme-depleted) wild type and site-directed mutant strains, we established that (i) the single immunoreactive band, observed in strains carrying the b_l site-directed mutations, corresponds to apocytochrome b_6 and (ii) the additional band present in strains carrying b_h site-directed mutations corresponds to a b_l-heme-dependent intermediate in the formation of holocytochrome b_6. Five nuclear mutants (*ccb* strains) that are defective in holocytochrome b_6 formation display a phenotype that is indistinguishable from that of strains carrying site-directed b_h ligand mutants. The defect is specific for cytochrome b_6 assembly, because the *ccb* strains can synthesize other *b* cytochromes and all *c*-type cytochromes. The *ccb* strains, which define four nuclear loci (*CCB1*, *CCB2*, *CCB3*, and *CCB4*), provide the first evidence that a *b*-type cytochrome requires trans-acting factors for its heme association.

Quinol oxidizing complexes, the cytochrome bc_1 complex of mitochondria and bacteria, and its chloroplast counterpart, the cytochrome b_6f complex, couple translocation of protons across the membrane to oxidation of lipophilic electron carriers (quinols) and reduction of small hydrophilic proteins (reviewed in Refs. 1 and 2).

In the green alga *Chlamydomonas reinhardtii*, the cytochrome b_6f complex comprises seven subunits. The four large subunits are in a 1:1:1:1 ratio (3, 4). Three of them are encoded by chloroplast genes (5), *petA* (encoding cytochrome *f*, a *c*-type cytochrome), *petB* (encoding cytochrome b_6), and *petD* (encoding subunit IV); whereas the Fe_2S_2 Rieske protein is encoded by a nuclear gene, *PetC* (6). In addition, the cytochrome b_6f complex contains three transmembrane subunits of low molecular mass (≤4 kDa), the products of the chloroplast genes *petG* (7–9) and *petL* (10) and the nuclear gene *PetM* (11, 12).

The cytochrome b_6f complex binds five cofactors: two b hemes (high potential b_h and low potential b_l), one c heme, a Fe_2S_2 cluster, and a chlorophyll *a* (13–16). In *c*-type cytochromes, the heme is covalently attached to the protein by thioether linkages between the sulfhydryl groups of (usually) two cysteine residues and the vinyl groups of the tetrapyrrole ring. In *b*-type cytochromes, the heme is believed to be noncovalently bound to the protein through pairs of histidines, which serve as axial ligands for the iron atom.

Cytochrome b_6 and subunit IV are homologous, respectively, to the first four α-helices and next three α-helices of cytochrome *b* of the cytochrome bc_1 complex (17). b_h and b_l hemes are associated with cytochrome b_6, b_l heme on the lumenal side of the thylakoid membrane (close to the quinol oxidizing site) and b_h heme on the stromal side (close to the quinone reducing site).

The crystallographic structure of the bc_1 complex from bovine heart mitochondria is being resolved (18, 19). The two hemes are bis-histidine-coordinated (20) and span the membrane bilayer approximately perpendicularly to the membrane plane (21). The two pairs of histidines that coordinate the central iron atoms are conserved in all cytochrome *b* (22) and located on helices B and D as follows in *C. reinhardtii*: (B)His_{100}-b_h-His_{202}(D) and (B)His_{86}-b_l-His_{187}(D) (5).

Numerous mutations of cytochrome *b* have been obtained in photosynthetic bacteria (*Rhodobacter* species) and mitochondria (reviewed in Ref. 23). By contrast, only a few mutations in *petB* that alter cytochrome b_6 are known (24–26). These mutations were generated in *C. reinhardtii*, which is a unique system for mutational studies of the cytochrome b_6f complex owing to the availability of chloroplast gene replacement methodology coupled with the fact that the cytochrome b_6f complex is dispensable for growth in *C. reinhardtii*.

In contrast to knowledge accumulated on the biosynthetic pathway of the tetrapyrrole cofactors and on the process of covalent attachment of *c* hemes to their apoproteins (reviewed in Refs. 27–30), conversion of *b*-type apocytochromes to their holo-form has received little attention, possibly due to the difficulty in distinguishing heme association defects from defects in other aspects of cytochrome *b* assembly. Since hemin can bind, without catalysis, to synthetic peptides that mimic helices B and D of cytochrome *b* (31), one view is that heme binding to cytochrome *b* should also be uncatalyzed *in vivo*. This view is uncontested by virtue of the fact that protein factors involved in the catalysis of heme binding to cytochrome

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b or b_6 have not been identified. The hemes of mitochondrial and bacterial cytochrome *b* are not detected after SDS-PAGE[1] of membranes or purified complexes (32, 33), and this undoubtedly accounts for the dearth of information on cytochrome *b* assembly. By contrast, heme(s) of cytochrome b_6 can be visualized after electrophoresis (3, 34–36). Here, we demonstrate that tight binding of heme is a unique aspect of the chloroplast cytochrome b_6 (which may reflect a different mode of association, perhaps covalent, of at least one of the two hemes), and we exploit this property to (i) establish a temporal order of heme binding to apocytochrome b_6 in a multistep pathway *in vivo* and (ii) identify mutants that are blocked specifically at the step of heme insertion into the b_h site. This has been accomplished by monitoring the synthesis of cytochrome b_6 in strains carrying site-directed alterations of b_l or b_h ligands and in nuclear mutants with similar phenotypes. These nuclear mutants define four loci.

MATERIALS AND METHODS

Strains—Wild type (WT) strain CC125 *mt+* was used for UV mutagenesis, and 137c was used for chloroplast transformation and genetic analysis. Strains were grown on Tris acetate phosphate (TAP) medium, pH 7.2, at 25 °C under dim light (5–6 μE).

Isolation of Nuclear Mutants Deficient in Cytochrome b_6f Complexes—WT cells grown to a density of 3–5 $\times$ 10^6 cells·ml^{-1} were transferred to Petri dishes exposed to 260-nm UV irradiation for 1–5 min with constant agitation. The cells' viability was approximately 20–30%. Irradiated cells were transferred to the dark for 2 h to minimize photoreversion. The cells were then plated on thin agar slabs (2% TAP agar poured over Miracloth circles) placed over TAP agar plates. Plates were returned to the dark for 24–48 h. Agar slabs were transferred to TAP agar plates containing 20 mM metronidazole and placed in high light (50–60 μE). After 24–48 h, agar slabs were transferred to fresh TAP agar plates and maintained under dim light for 2–3 weeks until colonies were apparent (50–150 colonies/plate). Surviving colonies were tested for fluorescence induction kinetics (3, 38); mutants showing a decreased cytochrome *f* by immunoblotting and a fluorescence of cytochrome b_6f-deficient mutants (26) were chosen.

Mutagenesis and Plasmids—Site-directed mutagenesis was performed in *Escherichia coli* as by Kunkel (39) on plasmid pdWB (24), which encompasses the whole *petB* coding sequence and its flanking regions. Mutated products were verified by sequencing. Plasmid pWFA was constructed by introducing the 1.9-kilobase pair *Eco*RV-*Sma*I fragment of plasmid pUC-atpX-AAD (40), bearing the *aadA* cassette, at the unique *Eco*RV site of plasmid piWF (24). In the resulting pWFA plasmid, the *aadA* cassette is located 309 base pairs downstream from the end of the *petA* coding region and is transcribed from the same strand as the *petA* gene.

Chloroplast Transformation Experiments—C. reinhardtii cells were transformed by particle bombardment as by Boynton *et al.* (41). We attempted to complement the Δ*petB* strain, deleted for the *petB* gene (24), with the mutated *petB* genes (Table I); after bombardment, transformant cells were plated on minimum medium (MM) under high light. Since no phototrophic transformants were recovered by the above procedure, we used the WT strain in co-transformation experiments with plasmid pWFA, which confers resistance to spectinomycin. Cells were grown for approximately six generations in the presence of 0.5 mM 5-fluorodeoxyuridine before transformation (42) and plated after transformation on TAP-spectinomycin (100 μg·ml^{-1}) under dim light to select for spectinomycin-resistant clones. Resistant clones were then screened by fluorescence to choose those that were defective in cytochrome b_6f activity. The transformants were confirmed to be homoplasmic for the *petB* mutation by restriction fragment length polymorphism analysis and DNA filter hybridization with specific probes (data not shown). The introduced mutations were further confirmed by direct sequencing of the mutated *petB* genes in these transformants (data not shown).

Genetic Analysis—Crosses were done according to Harris (43), and complementation analysis between nuclear mutants was done according to Goldschmidt-Clermont *et al.* (44). For reversion tests, mutant strains were grown in TAP to a density of 2 $\times$ 10^6 cells·ml^{-1}; the cells were collected by centrifugation and resuspended in MM to a density of 2 $\times$ 10^8 cells·ml^{-1}. One-half ml was spread onto MM agar plates (10 plates) and maintained under high light for 2–3 weeks, at which time colonies were counted. Recombination tests were performed to detect tight linkage; at the same time, at least 30 zygotes isolated from crosses between different mutant strains were transferred separately to either TAP or MM agar plates. They were kept under dim light on TAP plates during 2 weeks or high light on MM plates for 3–4 weeks; the number of zygotes giving rise to colonies was estimated on each plate; the number of tetratype and nonparental ditype tetrads was estimated as $a_1/(b_1 \times a_2/b_2)$, where a_1 is the number of zygotes that give rise to colonies on minimal medium, a_2 is the number of zygotes transferred to minimal medium, b_1 is the number of zygotes that give rise to colonies on TAP, and b_2 is the number of zygotes transferred to TAP.

Protein Isolation, Separation, and Analysis—Biochemical analyses were carried out on cells grown to a density of 2 $\times$ 10^6 cells·ml^{-1}. For analysis of polypeptide contents, samples were resuspended in 100 mM DTT, 100 mM Na_2CO_3 and solubilized in the presence of 2% SDS at 100 °C for 50 s. When indicated, nonheated samples were solubilized in the presence of 2% SDS at room temperature. Polypeptides were separated in the Laemmli system (45) using 12–18% acrylamide gels in the presence of 8 M urea or using 15% acrylamide gels containing 0.1% SDS. Heme staining was detected by peroxidase activity of heme binding subunits using 3,3′,5,5′-tetramethylbenzidine (TMBZ) as in (46). Immunodetection was carried out as in Kuras and Wollman (24) using a cytochrome b_6 antibody against an N terminus peptide alone that strongly labels a contaminant (Figs. 2*A* and 5*A*) or in combination with an antibody against a C terminus peptide that attenuates contamination (Figs. 3*B*, 6*B*, 7, and 8). Thylakoid membrane proteins were purified as in Ref. 47. Cytochrome complexes were extracted by hecameg solubilization of thylakoid membranes (4). For simplified membrane preparations, cells were harvested by centrifugation, concentrated to 15 $\times$ 10^6 cells/ml in 5 mM HEPES-KOH, pH 7.5, 10 mM EDTA, 0.3 M sucrose containing protease inhibitors (200 μM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 5 mM ϵ-amino-*n*-caproic acid), broken in a French press at 4000 p.s.i., and collected by centrifugation at 15,000 $\times$ *g* for 10 min. For phosphate/acetone extraction, the supernatant from hecameg-solubilized thylakoid membranes (4) was incubated in 500 mM ammonium phosphate, pH 8.0 (50 μl of supernatant added to 50 μl of 1 M NH_4HPO_4, pH 8.0) at 4 °C for 30 min and then extracted with 10 volumes of chilled 100% acetone for 10 min. For acid/acetone extraction, the supernatant from hecameg-solubilized membranes was incubated at 4 °C for 30 min in 20 volumes of chilled acetone containing 0.2% of HCl (12 N). The precipitating proteins after phosphate/acetone or acetone/acid treatments were collected by centrifugation and prepared for electrophoresis as above.

Pulse-labeling Experiments—Whole cells (2 $\times$ 10^6 cells·ml^{-1}) were pulse-radiolabeled for 5 min as by Delepelaire (48). ^{14}C-Acetate was used in the presence of 8 μg·ml^{-1} cycloheximide (an inhibitor of cytosolic protein synthesis) at 10^{-4} M (5 μCi·ml^{-1}). To end the labeling period, the isotope was diluted by the addition of 10 volumes of chilled sodium acetate (50 mM).

Gabaculine Inhibition of the Tetrapyrrole Biosynthetic Pathway—Cells grown to a density of 1.5 $\times$ 10^6 ml^{-1} were treated with 2 mM gabaculine for 6 h as by Howe *et al.* (49). Cells were harvested by centrifugation, washed, and concentrated to 1.5 $\times$ 10^7 cells ml^{-1} in minimum Tris medium with 1–2 mM gabaculine and agitated for 1 h. Cells were then pulse-labeled for 10 min with ^{14}C-acetate at a final concentration of 2 $\times$ 10^{-4} M (10 μCi·ml^{-1}) in the presence of cycloheximide (8 μg·ml^{-1}) and 2 mM gabaculine. For immunodetection of cytochrome b_6 after gabaculine treatment, cells were not pulse-labeled, and simplified membrane preparation was used.

RESULTS

Heme Binding to Cytochrome b_6 Is Partly Resistant to Denaturing Treatments—Since cytochrome b_6 can be heme-stained after electrophoresis (3), while the related cytochrome *b* cannot be, we tested denaturing treatments known to extract noncovalently bound hemes to determine the chemical basis for this unique property of cytochrome b_6. A cytochrome b_6f-enriched fraction obtained by hecameg solubilization of WT thylakoid membranes was treated with phosphate/acetone, with acid/acetone (50), or by boiling for 50 s in the presence of 2% SDS and analyzed for heme content after electrophoresis in urea-containing polyacrylamide gels. Surprisingly, cytochrome b_6

[1] The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; MM, minimum medium; TAP, Tris acetate phosphate medium; TMBZ, 3,3′,5,5′-tetramethylbenzidine; WT, wild type; E, einstein(s).

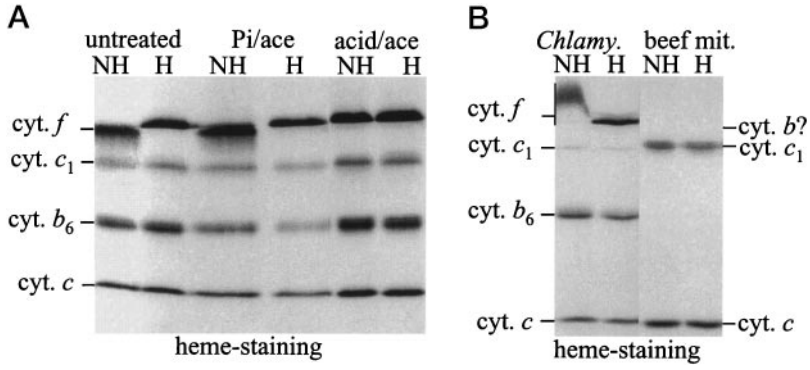

FIG. 1. **Cytochrome (*cyt.*) b_6 heme staining after various extraction treatments in WT and mutant strains.** *A*, TMBZ staining of supernatants of *C. reinhardtii* WT membranes solubilized with hecameg and analyzed by urea/SDS-PAGE. Samples were untreated or treated with phosphate/acetone or acid/acetone, not heated (*NH*) or heated (*H*) at 100 °C for 50 s. *B*, TMBZ staining of supernatants of *C. reinhardtii* WT membranes, solubilized with hecameg, and bovine heart mitochondrial membranes, analyzed by SDS-PAGE on a gel containing 15% acrylamide and 0.1% SDS. Samples were not heated (*NH*) or heated (*H*) in the presence of 2% SDS at 100 °C for 50 s. Cytochrome *b* is not visible by TMBZ. Its tentative migration position is indicated as *cyt. b?*.

continued to stain for heme by the TMBZ assay even after these treatments (Fig. 1*A*). To confirm that this property is unique to cytochrome b_6, we used a membrane preparation from bovine heart mitochondria to analyze the heme-cytochrome *b* interaction after electrophoresis on a 15% acrylamide gel containing 0.1% SDS (Fig. 1*B*). This gel system resolves the closely migrating mitochondrial cytochrome *b* and c_1 (33). As noted in that work, TMBZ staining revealed only one slowly migrating band, attributed to cytochrome c_1, and a high mobility band, typical of cytochrome *c*, with no evidence for cytochrome *b* staining. By contrast, cytochrome b_6 was heavily stained with TMBZ under these conditions (Fig. 1*B*). These experiments point to unusually tight binding of heme(s) to cytochrome b_6, *i.e.* resistant to denaturation. Since cytochrome b_6 did not stain with TMBZ more heavily than cytochrome *f*, which binds only one heme, we suggest that only part of the b heme complement of cytochrome b_6 is tightly associated with the polypeptide.

Mutation of the Heme-binding Histidines Revealed Distinct Forms of Cytochrome b_6 That Are Resolved by Gel Electrophoresis and Correspond to b_l Site and b_h Site Mutants—To determine whether we could distinguish which of the two hemes bound tightly to apocytochrome b_6 and whether both heme binding sites were required for cytochrome b_6 assembly, we used site-directed mutagenesis to construct *petB* genes in which one of the four heme-liganding histidines, either His_{100} and His_{202} to b_h or His_{86} and His_{187} to b_l, were substituted individually (Table I). At least three independent transformants for each modification were characterized for their biochemical properties. None of the transformants displayed a heme-stainable cytochrome b_6 after SDS-PAGE of solubilized membranes, and the level of heme-stained cytochrome *f* was reduced to that observed in a Δ*petB* deletion strain (data not shown). Immunodetection indicated that in each transformant cytochrome *f* accumulated to 10% of the WT level, and cytochrome b_6 and subunit IV were present in trace amounts (Fig. 2*A*).

Analysis of the immunoreactive species in the transformants *versus* the WT strain indicated a striking change in the migration pattern of cytochrome b_6 (Fig. 2*A*). Holocytochrome b_6 migrated as a broad diffuse band in the WT (Fig. 2*A*, *lane WT*, and dilution series in *bottom part*), as it does in Δ*petA* and in Δ*petD* mutants (*bottom part* of Fig. 2*A*), which synthesize holocytochrome b_6, while it migrated as discrete sharp bands in the *petB* site-directed heme-binding mutants. (The diffuse pattern in the WT strain does not result from overloading, because the same pattern is noted in dilute samples or in the Δ*petA* and Δ*petD* strains, which accumulate much less holocytochrome b_6.) The Δ*petB* lane serves as a control to indicate that the diffuse band in WT indeed corresponds to cytochrome b_6 and that a band, noted with an *asterisk*, is an unrelated cross-reacting signal. The contaminating polypeptide appears in the region of cytochrome b_6 only when electrophoresis is carried out in the presence of 8 M urea (Fig. 2*A*, *second part*). This cross-reacting band is not visible in the absence of urea (Fig. 2*A*, *third part*). In the four transformants bearing substitutions of the b_l heme ligands, cytochrome b_6 migrated as a single band below the contaminating *asterisk* band (Fig. 2*A*, *second part*, b_l transformants). By contrast, in the four transformants bearing substitutions of the b_h heme ligands, cytochrome b_6 migrated

TABLE I

Mutations introduced in the chloroplast petB gene of C. reinhardtii

In the first column, the four His coordinating regions in WT cytochrome b_6 are shown by the corresponding nucleotide sequences of the *petB* gene and their translation to the primary amino acid sequence (single letter code). Below are shown the various mutations at the His sites, with nucleotide changes shown in boldface type. The resulting substituted amino acid is shown in italics. Neutral mutations aimed at creation of new restriction sites are underlined in the sequence and named in the second column. The third column shows the names of the resulting mutated plasmid and the *C. reinhardtii* transformant strains carrying the mutations (indicated in parentheses).

Nucleotide and amino acid sequences	New restriction sites	Plasmid (Strain)
...ATT CAC CGT TGG...		WT
I^{85} H^{86} D^{87} W^{88}		
...**GCG CGG**...	*Bss*HII	pB86A
Ala		(TB86A)
...**TCG** CG**A**...	*Nru*II	pB86S
Ser		(TB86S)
...GTT TTA CAC GTT TTC...		WT
V^{98} L^{99} H^{100} V^{101} F^{102}		
...TTA **G**AC GT**C**...	*Aat*II	pB100D
Asp		(TB100D)
...TT**A** CT**A** GTT...	*Spe*I	pB100L
Leu		(TB100L)
...AGT CAC ACT TTC...		WT
S^{186} H^{187} T^{188} F^{189}		
...**GGT** AC**C**...	*Kpn*I	pB187G
Gly		(TB187G)
...**AGT** ACT...	*Sca*I	pB187S
Ser		(TB187S)
...CAC TTC TTA ATG ATT CGT AAA...		WT
H^{202} F^{203} L^{204} M^{205} I^{206} R^{207} K^{208}		
...**G**AC TTC TAA ATG AT**C** CG**G** AAA...	*Bsp*EI	pB202D
Asp		(TB202D)
...CA**G** TTC **CTG** ATG ATT CGT AAA...	*Alw*NI	pB202Q
Gln		(TB202Q)

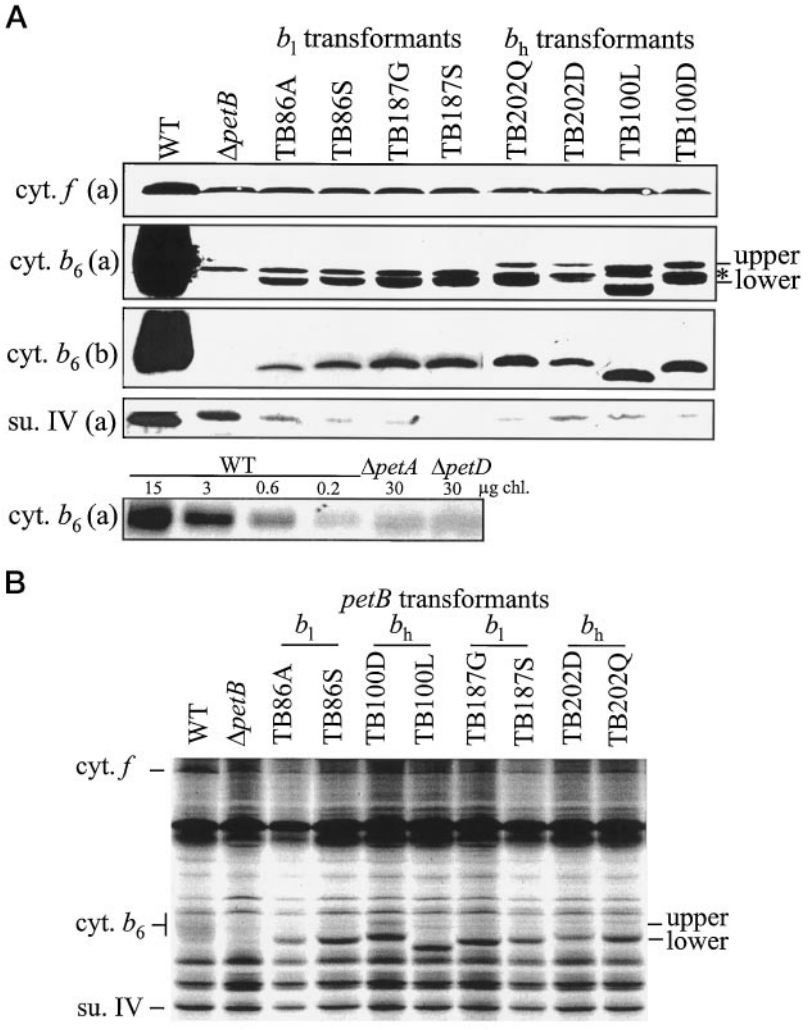

FIG. 2. **Accumulation and synthesis of cytochrome (*cyt.*) b_6f subunits in transformant strains.** *A*, content of cytochrome b_6f subunits analyzed by immunoblotting experiments. Whole cell polypeptides were separated on 12–18% acrylamide gels in the presence of 8 M urea (*a*) or in the absence of urea (*b*) blotted to nitrocellulose paper and revealed with specific antibodies for cytochrome *f*, cytochrome b_6, and subunit IV. The names of the strains are given with the heme ligands affected (b_l or b_h) in the *petB* transformants (see Table I). An unrelated cross-reaction of the b_6 anti-peptide antibody (also present in the mutant strain deleted for the *petB* gene (Δ*petB*)) is observed when the gel is run in the presence of urea, and its position is noted (*). When cytochrome b_6 migrated as a doublet (urea gels), the bands are referred to as *upper* and *lower*. Mutant strains deleted in the *petA* gene (Δ*petA*) or *petD* gene (Δ*petD*) show a diffuse cytochrome b_6 as in the WT strain. *B*, autoradiogram of a urea/SDS-PAGE gel showing chloroplast-encoded proteins radiolabeled for 5 min in the presence of ^{14}C-acetate and an inhibitor of cytoplasmic translation. Polypeptides of radiolabeled cells (amount equivalent to 15 μg of chlorophyll/lane) from WT and different mutant strains were separated by urea/SDS-PAGE.

as a doublet on both sides of the *asterisk* band (Fig. 2*A*, *second part*, b_h transformants); small variations in electrophoretic mobility of these bands were observed, depending on the substituting residue (Fig. 2*A*, see for instance b_h transformant TB100L). Thus, mutations preventing histidine ligation of the b_h hemes can be distinguished from those preventing b_l heme ligation, according to their migration pattern on SDS-urea gels. This suggested that it might be possible to resolve intermediates in the *in vivo* synthesis of holocytochrome b_6 from apocytochrome b_6 on the basis of their migration pattern in SDS and urea-containing polyacrylamide gels.

The synthesis of cytochrome b_6 was monitored by a brief period of labeling. In the WT strain, cytochrome b_6 appears as a broad and diffuse band similar in shape to that detected by immunoblotting or TMBZ staining (Fig. 2*B*). By contrast, the transformants display sharp and heavily labeled bands in the region of cytochrome b_6 migration, indicating that the rate of *petB* mRNA translation is not reduced greatly in the heme-binding site mutants. The absence of a comparable signal in the Δ*petB* strain confirms that the signal in the mutants corresponds to the *petB* translation product. The decreased accumulation of cytochrome b_6 in the heme-binding site mutants (Fig. 2*A*) must result from degradation of a protease-susceptible apoprotein. Accordingly, the rate of synthesis of cytochrome *f* in the mutants is reduced to about 10% of that observed in the WT strain, but no change in the rate of subunit IV synthesis is noted, which is consistent with the phenotype of the Δ*petB* deletion strain (24). In summary, the pattern of the cytochrome b_6 bands in the mutant strains were similar whether probed for accumulation (Fig. 2*A*) or synthesis (Fig. 2*B*).

Synthesis of Cytochrome b_6 in the Presence of Gabaculine, an Inhibitor of the Tetrapyrrole Biosynthetic Pathway, Reveals a b_l Heme-dependent Intermediate in the Biogenesis of Cytochrome b_6—To order the electrophoretic species identified in the strains carrying b_l *versus* b_h ligand mutations in the context of holocytochrome b_6 maturation, synthesis of cytochrome b_6 was monitored in cells depleted of heme. This was accomplished by treating cells with gabaculine, which prevents tetrapyrrole synthesis, for 6 h prior to and during a pulse-labeling experiment. Radiolabeled cells were solubilized and analyzed by urea/SDS-PAGE. In gabaculine-treated WT cells, neosynthesized cytochrome b_6 migrated as a discrete band at the front of the diffuse band observed in untreated cells (Fig. 3*A*) in a pattern reminiscent of that of radiolabeled b_l site mutants (see Fig. 2*B*). By contrast, the pool of cytochrome b_6 that had accumulated in the membranes and was preexisting was still visualized as a broad, diffuse band whether or not the cells were treated with gabaculine (Fig. 3*B*). Again, the absence of a signal in the Δ*petB* strain authenticates the identity of the bands in the WT and TB202Q strains. Thus, heme depletion by gabaculine treatment prevents heme assembly into neosynthesized apocytochrome b_6 but has no effect on preexisting cytochrome b_6. In gabaculine-treated b_l transformants (*e.g.* strain TB187G, Fig. 3*B*) we observed no change in the electrophoretic position of the cytochrome b_6 band. In contrast, in gabaculine-treated b_h transformants (*e.g.* strain TB202Q), most of the newly synthesized cytochrome b_6 doublet is converted to a discrete single band migrating in the position of the lower band

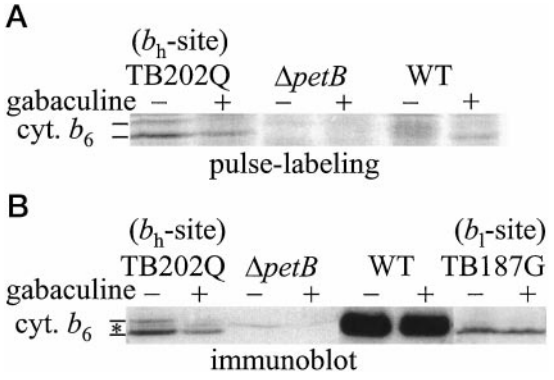

FIG. 3. **Gabaculine treatment prevents the formation of slowly migrating cytochrome (*cyt.*) b_6 species in the b_h site mutant.** Cytochrome b_6 was untreated (−) or treated (+) with gabaculine. Analysis by urea/SDS-PAGE is shown. *A*, synthesis of *petB* gene products in WT cells and cells of site-directed mutant TB202Q and deletion mutant Δ*petB*. The cells were radiolabeled in the presence of cycloheximide (8 μg/ml) and gabaculine (2 mM). *B*, immunodetection of protein accumulation in simplified membrane preparations with an antibody specific for cytochrome b_6. Membranes equivalent to 2.5 μg of chlorophyll were loaded for the untreated WT strain and 25 μg for the untreated mutant strains. The corresponding volume was loaded for the gabaculine-treated samples, which corresponds to a lower chlorophyll content due to the inhibition of the tetrapyrrole biosynthetic pathway by gabaculine.

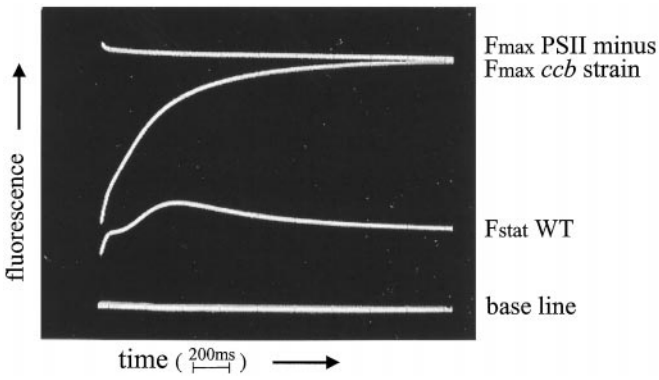

FIG. 4. **Typical fluorescence induction pattern of a *ccb* nuclear mutant.** The fluorescence induction pattern observed in a dark to light transition for a *ccb* mutant is shown in comparison with those from a WT and a typical photosystem II-deficient mutant. Note the continuously rising curve for the *ccb* strains, typical of a specific block in electron transfer at the level of cytochrome b_6f complexes. The strains were plated on TAP agar 3 days before the experiment.

of the doublet (Fig. 3*A*). Since the pool size of cytochrome b_6 accumulated in the mutant is small, reflecting the shorter half-life of the mutant protein, the effect of gabaculine treatment on the upper band is apparent even when unlabeled extracts are examined in immunoblotting experiments (Fig. 3*B*); specifically, the proportion of the upper band of the doublet is highly decreased relative to that of the lower band. These results confirmed that (i) the lower band of the doublet occurring in b_h site mutants corresponds to apocytochrome b_6 and (ii) the upper band, whose synthesis is strongly reduced upon gabaculine treatment, is a heme-dependent intermediate in the formation of holocytochrome b_6. We suggest a temporal order of heme association with apocytochrome b_6 such that the b_l heme is assembled prior to the b_h heme.

A Set of Nuclear Mutants of C. reinhardtii Presents the Same Phenotype as the b_h Transformants—To characterize the nuclear factors involved in the biogenesis of cytochrome b_6f complexes, we have isolated a number of nuclear mutants deficient in cytochrome b_6f activity (3, 51), all of which, including the *ccb* strains described below, displayed fluorescence characteristics typical of mutants with impaired cytochrome b_6f activity. In contrast to the WT strains, their fluorescence yield (26) rose continuously to an F_{max} level (Fig. 4). They were not altered in photosystem II, since this defect would cause a loss in variable fluorescence and a block at the F_{max} level (see the flat fluorescence trace that is observed in mutants lacking photosystem II reaction centers in Fig. 4). The collection of mutants was screened by TMBZ staining, for the loss of cytochrome b_6 and reduced accumulation of cytochrome *f*, and by immunoblotting.

The set of *ccb* nuclear mutants accumulated low amounts of distinct forms of cytochrome b_6, which are visualized after electrophoretic separation of solubilized membrane proteins (Fig. 5). These forms of cytochrome b_6, revealed either by immunodetection (Fig. 5*A*) or by autoradiography (Fig. 5*B*), correspond in all *ccb* mutants to the doublet, which is similar to that observed in transformants with altered b_h axial ligands. This suggests that the *ccb* strains are blocked at the same step at which the engineered b_h site mutants are blocked, *viz*. at the step of conversion of the b_l heme-dependent intermediate to the holocytochrome (see Fig. 9). The nuclear *Ccb* genes would then be proposed to encode factors required for the proper interaction of the *b*-type hemes with apocytochrome b_6 to produce holocytochrome b_6. To support this model, representative *ccb* mutants were treated with gabaculine to test whether the *ccb* strains would behave exactly like the b_h site mutants (representative example in Fig. 6). Indeed, gabaculine treatment inhibited the formation of the b_l heme-dependent intermediate (upper band of doublet), and this is apparent both in "pulse" radiolabeling experiments, where *de novo* synthesis of cytochrome b_6 is monitored (Fig. 6*A*, compare *plus lane* to *minus lane* for *ccb4–2*), and also when cytochrome b_6 accumulation is assessed by immunoblotting (Fig. 6*B*, note the depletion of the upper band corresponding to the b_l heme-dependent intermediate).

The b_l Heme-dependent Intermediate Is Resistant to Denaturing Treatments—In an attempt to assess the nature of the modification that gives rise to the b_l heme-dependent intermediate (upper band), crude membrane preparations from WT, Δ*petB*, TB187G, TB202Q, *ccb1–1*, and *ccb4–2* strains were treated with phosphate/acetone, which should release noncovalently bound hemes. The migration pattern of cytochrome b_6 species remained unchanged in all strains after phosphate/acetone treatment (Fig. 7). Therefore, neither the diffuse aspect of cytochrome b_6 in the WT strain nor the upper band of the doublet in b_h site-directed mutants and nuclear *ccb* mutants can be accounted for by noncovalent association of heme with the polypeptide. To test whether the b_l heme-dependent intermediate (upper band) was associated with heme, heme staining assays were conducted after separation of membrane proteins under less denaturing gel conditions, such as solubilization on ice with 1% SDS or 0.88% octyl-glucoside, 0.22% SDS and electrophoresis at 4 °C in the absence of urea. Nevertheless, we could not detect a heme-staining band in the region of cytochrome b_6 in any of the b_h transformants or the *ccb* mutants after SDS-PAGE, despite attempts to use more sensitive heme detection methods sensitive to 1% of the WT content in cytochrome b_6 (data not shown).

Genetic Analysis Shows That Four Nuclear Loci Are Required for the Conversion of the b_l Heme-dependent Intermediate to Mature Holocytochrome b_6—The number of loci represented by the *ccb* strains was determined by genetic analyses. Two nuclear mutants with similar phenotypes, MΦ30 and MΦ35, obtained by random integration of transforming DNA (37), were also included in this analysis, although they displayed a much lower fertility. In recombination tests, it is assumed that mutations in the same gene should be closely

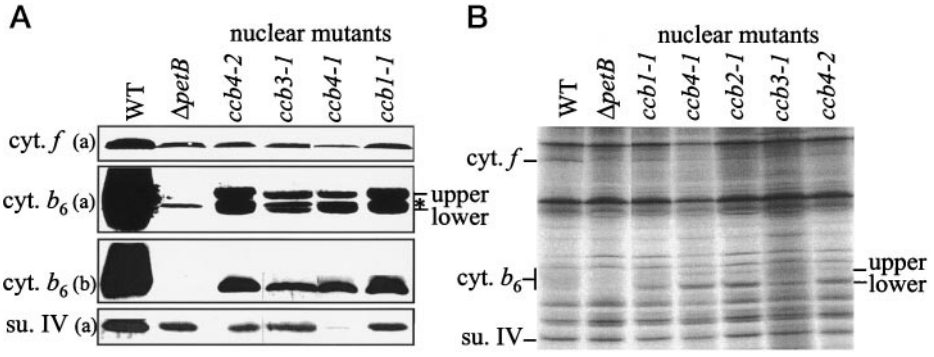

FIG. 5. **Accumulation and synthesis of cytochrome (*cyt.*) b_6f subunits in nuclear mutants.** *A*, content of cytochrome b_6f subunits analyzed by immunoblotting experiments as in Fig. 2*A*. The strains are identified by the alleles they carry at the nuclear *CCB* loci (see Table II). *B*, autoradiogram of a urea/SDS-PAGE gel showing synthesis of chloroplast-encoded proteins during a 5-min labeling in the presence of ^{14}C-acetate and an inhibitor of cytoplasmic translation as in Fig. 2*B*.

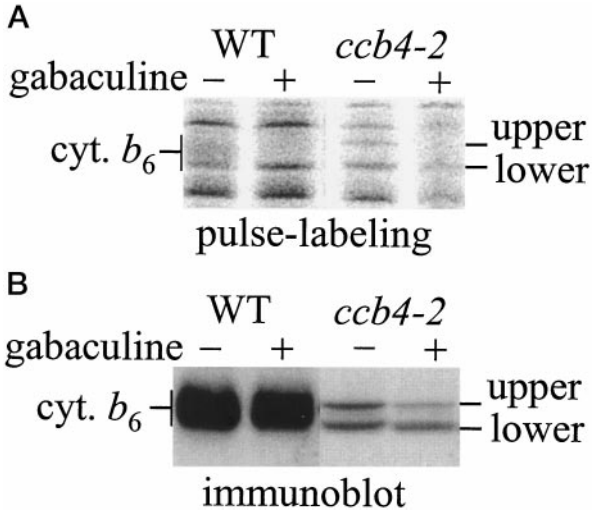

FIG. 6. **Gabaculine treatment prevents the formation of slowly migrating products of the *petB* gene in *ccb* mutants.** Cytochrome (*cyt.*) b_6 was untreated (−) or treated (+) with gabaculine. Analysis by urea/SDS-PAGE is shown. *A*, chloroplast-encoded protein synthesis in cells of WT and nuclear mutant *ccb4–2*. The cells were pulse-labeled in the presence of cycloheximide (8 μg/ml) and gabaculine (1 mM). *B*, immunodetection of cytochrome b_6 accumulation in simplified membrane preparations with an antibody specific for cytochrome b_6, as in Fig. 3.

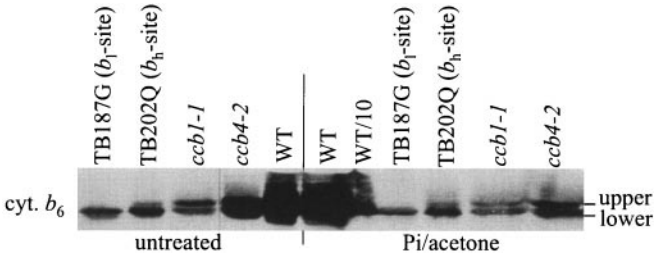

FIG. 7. **Migration pattern of *petB* gene products in WT and mutant strains treated with phosphate/acetone.** Migration pattern of cytochrome (*cyt.*) b_6 species in membrane preparations derived from WT and mutant strains, untreated (−), or treated (+) with phosphate/acetone. Polypeptides were revealed with an antibody specific for cytochrome b_6 after urea/SDS-PAGE.

linked, thereby preventing a high frequency of recombination events. The frequencies of tetratype and nonparental ditype tetrads were estimated from crosses between the various mutants (Table II, upper part). Three mutant strains gave rise to WT progeny when crossed with all of the other mutants, therefore each defining a locus, respectively *CCB1*, *CCB2*, *CCB3* (*C* for cofactor binding, *C* for cytochrome b_6f complex, and *B* for subunit PetB). Another three strains (*ccb4–1*, *4–2*, *4*-MΦ35) failed to recombine with each other but were able to recombine with the three mutants above. (The apparently lower recombination frequency for MΦ35 is probably due to its lower fertility.) Therefore, the three strains define alleles at a single locus, *CCB4*. We were unable to characterize further the locus of mutant MΦ30 because to its very low fertility. The conclusions from the recombination analysis were confirmed by complementation analysis (44). Only the two closely linked mutations in strains *ccb4–1* and *ccb4–2* failed to complement each other (Table II, lower part). The complementation tests confirmed that the two strains carried mutated alleles of the same gene. Thus, the genetic analysis of these six nuclear mutants define four different nuclear genes. Since the five mutations complement with at least three other mutations, they are all recessive mutations.

TABLE II

Complementation and recombination analysis of nuclear mutants affected in heme binding to cytochrome b_6

Complementation tests are shown below the diagonal (bottom and left side). Fluorescence analysis was performed on zygotes four days after mating. A plus sign indicates complementation detected as a restoration of a WT fluorescence pattern, while a minus sign indicates the absence of complementation in zygotes that retained a mutant fluorescence pattern. Recombination tests are shown above the diagonal (top and right side). Recombination was scored by measuring germination of zygotes on minimal medium after correcting for the viability of each cross (for details see "Materials and Methods"). Reversion frequencies are indicated in the boldface diagonal.

	ccb1–1	*ccb2–1*	*ccb3–1*	*ccb4–1*	*ccb4–2*	*ccb4*-MΦ35[a]
ccb1–1	**~7 × 10^{-6}**[b]	39/83	35/57	27/61	14/27	12/53
ccb2–1	+	**<~10^{-9}**	33/60	28/48	14/24	1/3
ccb3–1	+	+	**<~10^{-9}**	31/47	32/54	7/26
ccb4–1	+	+	+	**<~10^{-9}**	0/63	0/20
ccb4–2	+	+	+	−	**<~10^{-9}**	0/28
ccb4-MΦ35	ND[c]	ND	ND	ND	ND	**<~10^{-8}**

[a] Mutant strain *ccb4*-MΦ35 has lower frequencies of tetratype and nonparental ditype tetrads because of a strong mortality.
[b] Mutant strain *ccb1–1* has a tendency to revert.
[c] ND, not determined.

Analysis of Double Mutants Confirms That the ccb Strains Are Affected at the Same Step as Are the b_h Site Mutants, Which Follows the Step Affected in the b_l Site Mutants—To confirm the temporal order of cytochrome b_6 assembly suggested above (see Fig. 9 also), we generated double mutants by crossing the *ccb* strains with the chloroplast transformants lacking either a b_h or a b_l liganding histidine. The b_h TB202Q mt^+ transformant was crossed with each of five nuclear *ccb* mutant strains (*ccb1–ccb4*) mt^-, while the b_l TB187G mt^+ transformant was crossed with the nuclear mutants *ccb1–1* and *ccb4–2* mt^-. The resulting tetrads were dissected to recover the four progeny of the zygotes. All progeny had inherited the chloroplast $His^{202} \rightarrow Gln$ or $His^{187} \rightarrow Gly$ mutations, transmitted uniparentally by the mt^+ parent, while only two members of the tetrad inherited the nuclear mutant allele transmitted by the mt^- parent, the two other members having a WT nuclear genome. We analyzed the cytochrome b_6 content in the progeny from these crosses by immunoblotting. In all crosses, the four members of the tetrad presented the same phenotype, which was identical to the

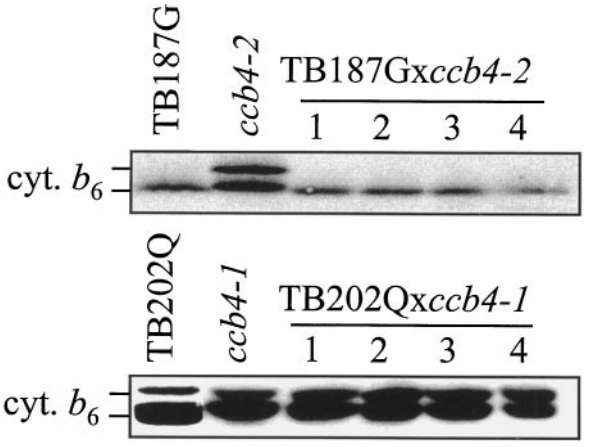

FIG. 8. **Cytochrome (*cyt.*) b_6 immunoblot of the four progeny of the tetrad derived from crosses between a b_l transformant (TB187G$^+$) and a nuclear mutant (*ccb4–2*$^-$) and between a b_h transformant (TB202Q$^+$) and a nuclear mutant (*ccb4–1*$^-$).** Polypeptides of whole cells were separated by urea/SDS-PAGE (as in Fig. 2).

phenotype of the chloroplast parent. Upon electrophoresis, cytochrome b_6 migrated as a doublet for the progeny of TB202Q × *ccb4–1* crosses, but it migrated as a single sharp band for the progeny of TB187G × *ccb4–2* crosses (Fig. 8). This confirms that the b_l site mutation affects a step before that affected in the *ccb* strains and that the phenotype conferred by the b_h site mutation is the same as that conferred by the mutations in the trans-acting *CCB* loci.

DISCUSSION

The unique denaturation-resistant association of heme with cytochrome b_6 suggested an unusual mode of b heme binding, and it also permitted us to dissect the b heme assembly pathway *in vivo* by exploiting genetic approaches in *C. reinhardtii*. Chloroplast mutants, obtained by site-directed mutagenesis of either one of the two histidines in each pair that coordinate b_h and b_l, were used as templates to define a distinctive phenotype for mutations affecting heme association with cytochrome b_6. Upon urea/SDS-PAGE, cytochrome b_6 migrates as a broad and diffuse band in the WT strain, whereas it is replaced by a sharp band corresponding to apocytochrome b_6 in b_l transformants and a distinct doublet in b_h transformants. The additional species observed in b_h transformants was identified as a b_l heme-dependent intermediate in the assembly of holocytochrome b_6. These characteristic features were used to identify a set of nuclear mutants that displayed the same phenotype as b_h site mutants. The mutants represent four nuclear loci, which we propose encode factors required specifically for the heme assembly into apocytochrome b_6.

Conversion of Apocytochrome b_6 to Holocytochrome b_6 Is a Multistep Process—We propose that the single, gabaculine-insensitive sharp band, detected in strains where the His ligands to b_l have been substituted, most likely corresponds to apocytochrome b_6, as noted previously for *Rhodobacter spheroides* bc_1 complex with substituted b_l ligands (52). In the case of the doublet detected in *ccb* mutants and in transformants mutated for the His ligands to b_h, the lower band is proposed to correspond to apocytochrome b_6 while the upper band is proposed to correspond to a b_l-dependent intermediate form. These assignments are supported by the results of radiolabeling experiments in the presence of gabaculine. When the heme pools are depleted, we expect that newly synthesized cytochrome b_6 will remain in the apoform, and the species detected under these conditions should correspond to apocytochrome b_6 (see for example, Ref. 53). Indeed, gabaculine-treated cells of the WT produce, upon short pulse labeling, a sharp band of the same electrophoretic mobility as that observed in untreated b_l transformants (*viz.* apocytochrome b_6). Also, the synthesis of the upper band of the doublet is hampered in *ccb* mutants and b_h transformants treated with gabaculine, which supports the model that the upper band represents a heme-dependent (perhaps heme-associated) species. The association of heme with the upper band could not be measured by heme staining. On the basis of its much reduced accumulation relative to holocytochrome b_6 (Figs. 3*B* and 6*B*), it is likely that the heme content of the upper band remains below the sensitivity of the staining technique. However, we cannot exclude the possibility that some post-translational modification accompanies b_l binding and yields this intermediate form of cytochrome b_6. The generation of this species is not dependent upon the *Ccb*-encoded factors, since the progeny derived from crosses between the b_h chloroplast transformant TB202Q and the various *ccb* mutants still display the electrophoretic doublet.

By analyzing the electrophoretic mobility of cytochrome b_6 in the WT strain *versus* those containing substitutions of the His ligands to b_l and b_h, we conclude that the diffuse aspect of WT cytochrome b_6 results from its interaction with the two hemes. Proper folding of the polypeptide in the environment of the b_h site is required for formation of the diffuse migrating species. For instance, substitution of Leu^{204} by a proline residue in the vicinity of His^{202} (b_h ligand) yields an electrophoretic doublet, presumably due to misfolding of the b_h attachment site (26).

The *ccb* mutants and the b_l/b_h chloroplast transformants are nonphototrophic strains and are deficient in the various subunits of the cytochrome b_6f complex. Since the abundance of *petB* transcripts and the rate of synthesis of cytochrome b_6 polypeptides were not decreased in these strains, their low content of cytochrome b_6 polypeptides must result from their increased proteolytic susceptibility because of impaired b heme binding.

Conversion of Apocytochrome b_6 to Holocytochrome b_6 Is a Specifically Assisted Process: Mutations in Four Different Nuclear Genes Result in the Same Phenotype as b_h Transformants—The five *ccb* mutants that display a phenotype identical to that of b_h transformants are altered specifically in the biosynthesis of cytochrome b_6 and not in the general pathway of b-type heme biosynthesis. The argument for this conclusion is as follows: (i) the *ccb* mutants are not deficient in cytochrome b_{559}, since they exhibit fluorescence induction curves characteristic of cytochrome b_6f mutants, whereas cytochrome b_{559} mutants, because they do not accumulate photosystem II (54), should have no variable fluorescence; (ii) holocytochrome *f* formation is normal (*i.e.* it stains for heme) in *ccb* mutants although its abundance is reduced due to disruption of assembly of the cytochrome b_6f complex; (iii) the synthesis and accumulation of cytochrome c_6 occurs normally in these strains (data not shown); and (iv) the mitochondrial cytochromes are present as evidenced by normal TMBZ staining of the *c*-type cytochromes and normal growth by dark respiration on TAP (data not shown). We therefore conclude that the *ccb* mutants are affected in nuclear factors specifically required for the proper binding of the *b*-type hemes to apocytochrome b_6 or for the folding of the apoprotein in a conformation suitable for heme association.

Genetic analysis indicates that the mutations belong to four nuclear loci, *CCB1*, *CCB2*, *CCB3*, and *CCB4*. Since three of the four complementation groups are represented by only one mutation, it is likely that the number of loci involved in the process may be higher. The number of nuclear genes involved in this single assembly process may seem rather high; the biochemis-

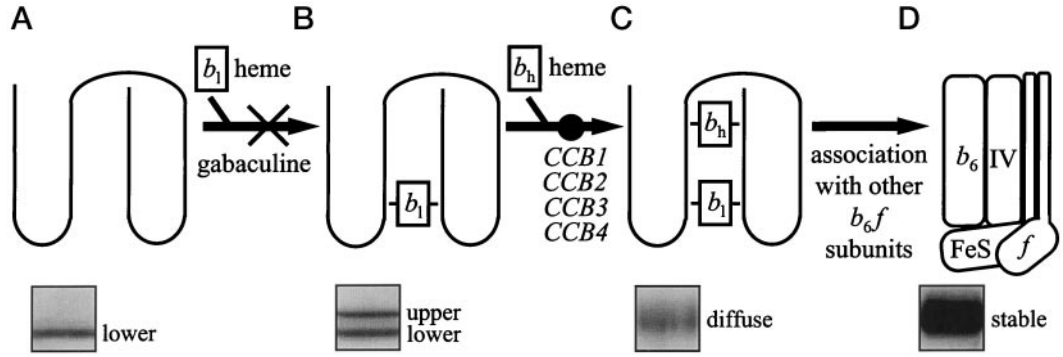

FIG. 9. **Schematic pathway of the conversion of apocytochrome to holocytochrome b^6 and patterns after urea/SDS-PAGE.** *A*, membrane integration occurs even in absence of heme association. *B*, there is formation of a b_l heme-dependent intermediate, which can be prevented by heme depletion (gabaculine treatment). *C*, *Ccb1–Ccb4* encoded nuclear factors are necessary for the production of the holo-form showing both b_h heme binding and b_l binding. *D*, holocytochrome b_6 accumulates in a protease-resistant form, upon association with the other b_6f subunits.

try of this process is not understood in any system, and it is therefore not possible to speculate on the function of these loci. In *Saccharomyces cerevisiae*, at least two loci (*CBP3* and *CBP4*) have been identified as encoding candidate cytochrome *b* assembly factors, but the inability to distinguish heme association mutants from those affected at an early step in bc_1 complex assembly has precluded a definitive functional assignment to these loci (55, 56).

One Heme May Be Covalently Attached to Cytochrome b_6—Surprisingly, denaturing conditions, such as acid/acetone treatment used to prepare globin from hemoglobin (50), which are generally considered to dissociate noncovalently bound hemes from proteins, did not affect the shape of the electrophoretic bands for cytochrome b_6; neither the WT diffuse band nor the doublet in b_h transformants or in *ccb* mutants was converted to a sharp single band. We could show, by TMBZ staining after urea/SDS-PAGE, that WT cytochrome b_6 retained b heme after phosphate/acetone or acetone/acid extraction. Heme binding to cytochrome b_6 also resisted boiling SDS. It should be noted, however, that it did not heme-stain more than cytochrome *f*, which binds only one heme. This observation suggests that only part of the b heme complement of cytochrome b_6 shows high binding affinity for the apoprotein.

The stability of a hemoprotein form of cytochrome b_6 is in sharp contrast with the various reports that b hemes are lost after SDS-PAGE of bacterial cytochrome *b* (32), mitochondrial cytochrome *b* (Ref. 33 and Fig. 1*B*), or the *b*-type cytochrome from *Chlorobium limicola*, which shares intermediate properties between cytochrome *b* and cytochrome b_6 (57). The unusual stability of *b* heme binding to cytochrome b_6 strongly suggests some linkage that cannot be formed within cytochrome *b* from *bc* complexes. Some residues close to the His ligands of the b_6 hemes, conserved in sequences of cytochrome b_6, but absent from cytochrome *b*, including the one from *C. limicola*, could be involved in interactions with the tetrapyrrole ring. Given the similar absorption spectra of cytochrome b_6 and cytochrome *b*, any covalent linkage of cytochrome b_6 hemes ought to occur through atoms that are not conjugated with the macrocycle. Site-directed mutations of residues that are candidates for providing covalent ligands to the hemes in the vicinity of b_l or b_h should allow us to settle this point.

Pathway from Apocytochrome b_6 to Holocytochrome b_6—From the various electrophoretic patterns of cytochrome b_6 forms, it seems reasonable to suggest that the products of the *CCB* loci catalyze the association of the b_h heme to the b_l-binding intermediate form of cytochrome b_6. A schematic view of the apo- to holo- conversion for cytochrome b_6 is presented in Fig. 9 with the following steps: membrane integration of apocytochrome *b* (*A*), formation of a b_l heme-dependent intermediate (*B*), b_h heme binding to b_l-dependent intermediate requiring *Ccb*-encoded trans-acting factors, perhaps responsible for the tight heme-binding, but occurring independently of the other b_6f subunits (*C*), concerted accumulation with the other subunits (*D*).

It may sound paradoxical that the *CCB* loci-assisted step is at the level of b_h binding to the b_l intermediate form. The latter is stable in denaturing conditions, thereby supporting a tight heme binding of b_l rather than b_h to cytochrome b_6. This raises the question of whether the form of cytochrome b_6 revealed in the b_h and *ccb* mutants is a genuine assembly pathway intermediate in cytochrome b_6 biogenesis or perhaps a dead end process that occurs only in the mutants. In this view, proper covalent binding of b_l to the apocytochrome may require the concerted presence of the b_h substrate and the *Ccb* gene products. In the absence of either of these factors, spontaneous covalent binding of b_l in an inappropriate conformation may occur at low yield generating the b_l-dependent intermediate. Regardless, the occurrence of this form has permitted us to resolve the two steps in holocytochrome b_6 formation.

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